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Materials and Methods

(1) AMCHA was Amikapron® (Kabi SA, Stockholm) (mixture of the active and inactive isomers).

(2) PAMBA® was supplied by Arzneimittelwerke Dresden.

(3) εACA was Epsilarpron® (Kabi SA, Stockholm).

(4) Streptokinase was Streptase® (Behring Werke, Marburg)

(5) Trasylol® 5000 KIE/ml (Bayer Leverkusen) molecular weight 11.600 (15), 1 U = 0,14 μg or 8.3×10^{-6} units/Mol.

(6) Plasminogen enriched ^{125}J labelled standard clots were prepared from fresh human plasma with labelled human fibrinogen and human plasminogen added according to Dubois de Wit (8). These clots were incubated 60 min in the test solutions at 37° C, the residual clot was taken out, and the activity in the solution was counted. The test solution was prepared by mixing 1.6 ml 0.9% NaCl, 0.2 ml Streptase (const. or variable concentration) and 0.2 ml of inhibitor (const. or variable concentration). 0.5 ml of the mixture were used to incubate the clot.

A blank was prepared by incubation of the clot in mixture of 1.6 ml 0.9% NaCl and 0.4 ml barbital buffer 100% lysis was the difference of counts in the clot before incubation and the blank. The results are expressed as percent lysis corrected for activity in the blank.

$$\frac{(\text{Test sample} - \text{blank})}{(\text{Clot} - \text{blank})} \times 100.$$

In the in vivo test the standard clots were incubated with 0.5 ml of the patient platelet poor citrated plasma.

(7) Englobulin lysis time (12)

(8) Casein test according to Norman (14) was used for the determination of the factors of the fibrinolytic system during the in vivo experiments. Euglobulin precipitates were used to avoid the influence of the inhibitors on the determinations. Plasminogen was determined by addition of 200 U/SK/ml, plasmin without any addition, and activator by addition of 10 U of human plasminogen.

(9) One stage prothrombin time.

(10) Partial thromboplastin time (9).

(11) Thrombin time with 5 U/ml Thrombin, normal value 8–13 sec.

(12) Fibrinogen determination Vermylen' modification of the method of Clauss (16)

(13) The synthetic inhibitors were qualitatively determined by two-dimensional paper chromatography (courtesy of Dr Cloos).

(14) Human plasminogen was prepared from Cohn fraction III according to the method of Kline.

Results

In vitro Tests

(1) In the first series of experiments SK was kept constant with 10 U/ml and the concentration of inhibitors was varied the synthetic inhibitors between 3×10^{-3} M and 5×10^{-1} M Trasylol between 0 1 U/ml and 10 U/ml ($1.2 \times 10^{-}$ to 1.2×10^{-7} M) final concentration (Fig 1) It was found that on a molar basis Trasylol is extremely more active than all the synthetic materials The difference is about 10^4

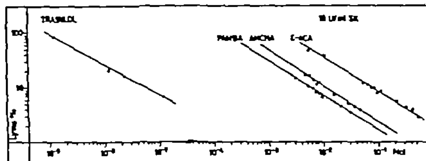


Fig 1 Action of inhibitors on clot lysis *in vitro*. Concentration of SK constant 10 U/ml, concentration of inhibitors variable. Ordinate log % residual lytic activity (^{131}J labelled standard clots) Abscissa log final molar concentration of the inhibitors.

Compared with this large difference, the differences between the synthetic inhibitors are small. PAMBA is about 2 times as active as AMCHA, and about 12 times as active as εACA.

(2) In a second series of experiments SK concentration was varied between 1 and 100 U/ml and the concentration of the synthetic inhibitors was kept constant at 1.25×10^{-3} and Trasylol at 1.2×10^{-6} and 1.2×10^{-7} M. In these experiments, residual activity (% lysis) is not calculated by referring to the maximal possible activity but to the activity obtained with the respective SK-concentration without inhibitor

About the same relationship of the activities of the 3 synthetic inhibitors was found as in the first experiment. This relationship is independent of the concentration of SK used, whereas the absolute degree of inhibition increases with reduction of the concentration of SK. Trasylol is extremely more active (Fig 2)

In vivo Experiments

(1) Fibrinolytic activity was induced in human volonteers by injection of 1 000 000 U SK within 30 min and sustained by 100 000 U/h for 3 ½ h. The inhibitors were injected intravenously at the end of the infusion. The effect was tested with the standard clot method 5 and 30 min after injection. 3.1×10^{-3} M AMCHA caused a 60 % inhibition of the fibrinolytic activity within 5 min whereas 3.8×10^{-3} M εACA were necessary to induce about the same inhibition, exactly 75 %. 6.6×10^{-4} M PAMBA caused a 20 percent inhibition and $6 \times 10^{-}$ M (50 000 U) Trasylol a 87 % inhibition (Fig 3) A second

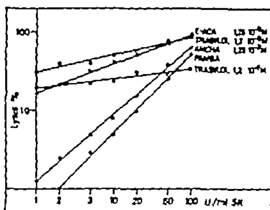


Fig. 2. Action of inhibitors on clot lysis *in vitro*. Concentration of synthetic inhibitors 1.25×10^{-5} M, (———) and Trasylol® 1 and 10 U (1.2×10^{-4} M and $1.2 \times 10^{-}$ M) final concentration constant during the experiment, Streptokinase variable. Ordinate: log % residual lysis (corrected for the lysis by streptokinase without inhibitor). Abscissa: log Units Streptokinase, final concentration.

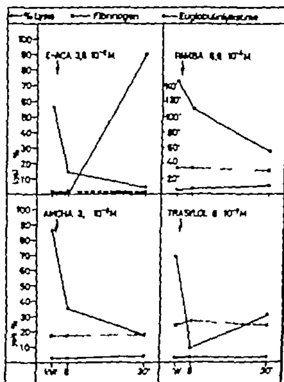


Fig. 3. Interruption of induced fibrinolysis by protease inhibitors. Abscissa: % lysis (^{125}J labelled clots), mg % fibrinogen or minutes euglobulin lysis time. Ordinate: Time after injection of the inhibitor. ●—● % Lysis, × mg % Fibrinogen, o— Euglobulin lysis time.

test was performed 25 min later The fibrinolytic activity was further reduced in the patients who received the synthetic inhibitors, whereas the fibrinolytic activity increased again from 9 % lysis to 31 % lysis after Trasylol. The euglobulin lysis time did not change 5 min after the injection of the inhibitors and was increased a little after 30 min The clotting tests were not influenced by the inhibitors in this short time period (Table I)

(2) In a second series of experiments 1 000 000 U SK were injected simultaneously with the inhibitors within 30 min The infusion was continued with 100 000 U SK/h. In the control experiment, in which SK without inhibitor was infused fibrinogen was reduced to 14 mg % within 30 min and remained on a level of about

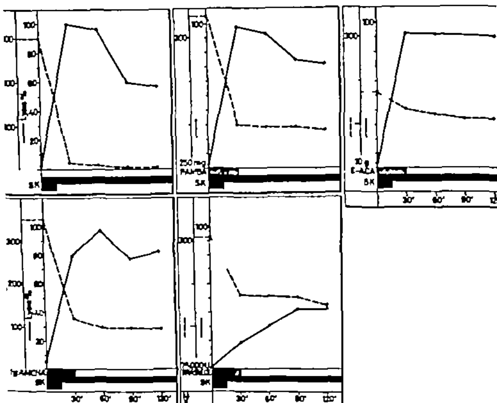


Fig 4 Simultaneous application of streptokinase and inhibitors. Concentration stated in the figures. ●—● % Lysis o—o mg % Fibrinogen. Time in minutes after starting the infusion.

Table I

Interruption of streptokinase-induced fibrinolytic activity *in vivo* by fibrinolysis inhibitors.

a) Clotting tests.

Treatment	Test system	Before	5 min after	30 min after
3.8×10^{-4} M	prothrombin	18%	19%	21%
εACA	PTT	>120 sec	>120 sec	>120 sec
	thrombin time	>90 sec	>90 sec	>90 sec
	fibrinogen	<1 mg%	6 mg%	3.5 mg%
	euglobulin lysis time	no clot	no clot	180 min
6.6×10^{-4} M	prothrombin	41%	36%	36%
PAMBA	PTT	85 sec	84 sec	62 sec
	thrombin time	26 sec	28 sec	26 sec
	fibrinogen	87 mg%	85 mg%	75 mg%
	euglobulin lysis time	9 min	no clot	no clot
3.1×10^{-4} M	prothrombin	47%	52%	54%
AMCHA	PTT	100 sec	90 sec	94 sec
	thrombin time	18 sec	18 sec	18 sec
	fibrinogen	85 mg%	87 mg%	89 mg%
	euglobulin lysis time	6 min 30 sec	5 min	8 min
$6 \times 10^{-}$ M	prothrombin	50%	52%	50%
Trasylol®	PTT	74 sec	69 sec	73 sec
	thrombin time	14 sec	13.4 sec	13.8 sec
	fibrinogen	118 mg%	132 mg%	118 mg%

10 mg% for the following period of 3 h. In correlation to this thrombin time and PTT were unmeasurable and prothrombin time below 20%. The J-labelled standard clot was completely lysed. The euglobulin precipitate did not clot. Plasminogen and plasmin disappeared and a large amount of activator was found (Fig. 4, Table IIb).

In the experiments with simultaneous application of streptokinase and 7.6×10^{-3} M εACA, 1.65×10^{-3} M PAMBA or 6.2×10^{-4} M AMCHA respectively a different behaviour of the clotting factors and not very much change in the reaction of the fibrinolytic system could be observed (Fig. 4, Table II). The three synthetic inhibitors behaved similarely, therefore the result may be discussed together. The fibrinolytic activity measured with the ^{125}J-labelled clots reached values between 85 and 98% lysis, the fibrinogen level remained between 80 and 1?0 mg%. In correlation prothrombin time was not reduced below 46%, thrombin time did not increase to more than ?6 sec and PTT not more than to 98 sec (Fig. 4, Table IIa).

Table I

b) Fibrinolysis tests. Casein units.

Treatment		Before	after 5 min	30 min
Plasminogen	3.8×10^{-4} M εACA	0	0	0.084
	6.6×10^{-4} M PAMBA	0.204	0.222	0.222
	3.1×10^{-4} M AMCHA	0.15	0.42	0.28
	6×10^{-7} M Trasylol®	0.14	0.111	0.231
Plasmin	3.8×10^{-4} M εACA	0	0.093	0
	6.6×10^{-4} M PAMBA	0.056	0.046	0.046
	3.1×10^{-4} M AMCHA	0.073	0.14	0.023
	6×10^{-7} M Trasylol	0.24	0.22	0.167
Activator	3.8×10^{-4} M εACA	6.38	6.25	3.14
	6.6×10^{-4} M PAMBA	9.17	8.39	5.086
	3.1×10^{-4} M AMCHA	6.14	5.71	4.24
	6×10^{-7} M Trasylol	6.29	5.76	6.31

The euglobulin precipitates clotted always and were lysed within 1 to 15 min. There was no influence on the disappearance of plasminogen and plasmin and on the formation of activator (Table IIb) 9 × 10− M (75 000U) Trasylol inhibited the lysis of the standard clot much more than the synthetic inhibitors. 1 h after terminating the trasylol infusion only 52 % lysis of the standard clot was observed. Fibrinogen disappeared much slower and remained on about 150 mg %. Prothrombin time was reduced from 75 % to about 50 %, PTT and thrombin time were not significantly influenced (Table IIa) There was a higher amount of plasminogen and plasmin left after 30 min. The activator activity was the same as in the other experiments (Table IIb)

The synthetic inhibitors were traceable in the serum for 2 to 4 h after the injection with εACA as longest.

Discussion

The activity of the inhibitors was tested *in vitro* in a system where plasminogen was activated by SK, and human fibrin was the substrate for the plasmin formed. It acted from outside and was not incorporated into the clot. The synthetic inhibitors differed not very much in activity εACA, the substance longest known, had the lowest activity AMCHA is about 6 to 8 times as active as εACA on a molar basis and PAMBA 12 to 14 times. If one consideres that AMCHA contains only 15 to 20 % of the active isomere, AMCA is more potent

Table II

Simultaneous administration of streptokinase and inhibitors.

a) Clotting tests.

Treatment	Method	Before	Time after starting infusion: 30 min	1 h	1 h 30 min	2 h	2 h 30 min
SK	Prothrombin	85%	<10%	17%	17%	20%	18%
	PTT	60 sec	>120 sec	>120 sec	>120 sec	>120 sec	>120 sec
	Thrombin time	13 sec	>90 sec	>90 sec	>90 sec	>90 sec	>90 sec
	Fibrinogen	300 mg%	14 mg%	8 mg%	6 mg%	11 mg%	<1 mg%
	ELT	12 h	no clot	no clot	no clot	no clot	no clot
SK +	Prothrombin	76%	63%	63%	63%	55%	54%
7.6×10^{-4} M	PTT	60 sec	71 sec	74 sec	73 sec	76 sec	73 sec
ε ACA	Thrombin time	12 sec	16 sec	16 sec	15.8 sec	15.4 sec	16 sec
	Fibrinogen	213 mg%	135 mg%	122 mg%	112 mg%	122 mg%	115 mg%
	ELT	12 h	15 min	16 min	13 min	13 min	11 min
SK +	Prothrombin	87%	48%	37%	37%	41%	41%
1.65×10^{-4} M	PTT	54 sec	79 sec	84 sec	77 sec	74 sec	79 sec
PAMBA	Thrombin time	12 sec	23 sec	24 sec	23 sec	26 sec	26 sec
	Fibrinogen	384 mg%	100 mg%	96 mg%	95 mg%	85 mg%	80 mg%
	ELT	5 h 18 min	<1 min	9 min	<1 min	8 min	9 min
SK +	Prothrombin	80%	56%	46%	48%	53%	52%
6.2×10^{-4} M	PTT	68 sec	83 sec	95 sec	98 sec	76 sec	94 sec
AMCHA	Thrombin time	13 sec	18 sec	17 sec	17 sec	17 sec	17.6 sec
	Fibrinogen	350 mg%	120 mg%	99 mg%	89 mg%	95 mg%	90 mg%
	ELT	>12 h	12 min	6 min	5 min	5 min	5 min
SK +	Prothrombin	78%	50%	51%	50%	50%	50%
9×10^{-7} M	PTT	75 sec	75 sec	77 sec	67 sec	76 sec	69 sec
Trasylol®	Thrombin time	12 sec	13 sec	14 sec	13 sec	14 sec	13.6 sec
	Fibrinogen	310 mg%	172 mg%	170 mg%	108 mg%	140 mg%	150 mg%

than PAMBA. This is in good agreement with the results of Dubber (6) who found AMCHA 2 times as active as εACA *in vivo* and 6 times as active *in vitro* with fibrinogen as substrate and activation of the fibrinolytic system with UK. Andersson (2, 3) found AMCA 6 times as active as εACA in the same system and 10 times as active with tissue activators. Markwardt (13) found PAMBA 2 times and AMCA 4 times as active as εACA with plasmin. For practical purposes one may conclude, that PAMBA and AMCHA are 10 times as active as εACA on a weight basis.

Trasylol is on a molar basis 10^3 to 10^4 times more active than the synthetic inhibitor. Its mode of action differs from that of the synthetic inhibitors, which is demonstrated by the different slope of the lines in Fig 1 and 2. This higher activity of Trasylol on molar

Table II

b) Fibrinolysis tests. Casein units.

	Treatment	Before	Time after starting infusion 30 min	60 min	90 min	120 min	150 min
Plasminogen	SK	5.25	0.093	0.13	0.056	0	0
	SK + 7.6 × 10^{-4} M εACA	3.51	0.159	0	0	0.37	0.063
	SK + 1.65 × 10^{-4} M PAMBA	4.4	0.18	0.055	0.305	0.26	0.13
	SK + 6.2 × 10^{-4} M AMCHA	4.35	0.28	0.046	0	0.36	0.046
	SK + 9 × 10^{-7} M Trasylol®	3.72	0.305	0.185	0.13	0.35	0.093
Plasmin	SK	0	0.074	0.111	0	0.074	0
	SK + 7.6 × 10^{-4} M εACA	0	0.057	0.112	0	0	0.033
	SK + 1.65 × 10^{-4} M PAMBA	0	0.13	0.063	0.046	0.083	0.18
	SK + 6.2 × 10^{-4} M AMCHA	0	0.28	0.057	0	0.23	0.13
	SK + 9 × 10^{-4} M Trasylol	0	0.41	0.16	0.32	0.1	0.222
Activator	SK	0	6.64	6.30	6.23	4.84	6.38
	SK + 7.6 × 10^{-4} M εACA	0	8.05	7.72	8.01	8.02	8.33
	SK + 1.65 × 10^{-4} M PAMBA	0	8.70	8.26	8.80	8.54	8.89
	SK + 6.2 × 10^{-4} M AMCHA	0	6.23	6.12	6.39	5.38	5.30
	SK + 9 × 10^{-7} Trasylol	0	5.996	6.33	6.23	6.33	6.42

basis has already been mentioned by DUBBER (7) and is in good agreement with their results.

The *in vivo* experiments demonstrate that it is easy to interrupt the fibrinolytic activity induced by an infusion of streptokinase with the synthetic inhibitors as well as with Trasylol. The fibrinolytic activity was significantly reduced 5 min after injection of the synthetic inhibitors and declined more in the following 30 min. This reduction of activity was not a consequence of stopping the streptokinase infusion. This is demonstrated by the fact, that the euglobulin lysis time was not affected after 5 min, and only a little prolonged after 30 min. This small prolongation is the consequence of the reduction of the level of the activator by excretion of streptokinase. Trasylol was in our experiment more active than the synthetic inhibitors, but the effect was of shorter duration. The lytic activity had reached half the pre-injection value again after 30 min.

If the absolute amounts of the inhibitors used are compared, the same relation becomes evident than in the *in vitro* experiments. 3.8 × 10^{-4} M εACA were a little but more active than 3.1 × 10^{-4} M AMCHA which means again an about 6 to 8 times greater activity of AMCHA on a molar basis or a 10 times greater activity on weight basis. PAMBA had to be used in a smaller dose because not enough injectable material was available. The dose was 5 times and the

inhibition about 3 times smaller than that of AMCHA. The *in vitro* experiments have demonstrated a linear relationship between concentration of the inhibitor and inhibition. Therefore, the conclusion may be justified, that PAMBA is about two times as active as AMCHA *in vivo*. 9×10^{-7} M Trasylol is as active as 3.8×10^{-2} M εACA, which means that Trasylol is about 50 000 times as active as εACA on a molar basis, but the action is of shorter duration. The injection of Trasylol has to be repeated in intervals of 15 to 30 min if the effect should be sustained.

About the same relation of activities is found, if the inhibitors are administered simultaneously with the streptokinase to protect the patient against the effect of overshooting plasminemia.

Verstraete (17) has recommended to induce streptokinase treatment with a constant dose of 1 250 000 U and to continue it with 100,000 U/h. He has three arguments in favour of this method (1) The dose is sufficient in at least 95% of the population. The determination of streptokinase resistance before treatment is unnecessary (2) The larger the dose of streptokinase the faster the blood will be depleted of plasminogen and the shorter is the duration of plasminemia (3) Afibrinogenemia was not noted in these experiments. Verstraete, however has obviously not determined fibrinogen levels in the first hours after starting the infusion. If the streptokinase resistance of the patient is low as f.i. in our control case (50 000 U) an afibrinogenemia will result at least in the first 4 h. This is one reason why ways must be found to avoid this situation. Another point is that streptokinase in excess of plasminogen will preferably induce the formation of activator and less that of plasmin. If we could block temporarely the action of the activator by an inhibitor without interfering with its formation plasminogen should be exhausted without formation of larger amounts of plasmin. This was the reason for trying to infuse large doses of streptokinase simultaneously with inhibitors. It was expected, that plasminogen should be transformed to activator After stopping the infusion of the inhibitor and continuing the infusion of streptokinase streptokinase will always be in excess of the newly formed small amount of plasminogen, and large amounts of activator but only negligible amounts of plasmin will continuously be found in the circulation. The excretion of the inhibitors is fast and the concentration of the activator is increasing and it will soon overcome the reduced concentration of the inhibitor The activator will become active in the thrombus.

Under these conditions it should be possible to reduce the effects of plasminemia to a minimum.

Our experiments seem to prove this hypothesis. When a large dose of streptokinase is infused simultaneously with a synthetic fibrinolysis inhibitor and the infusion of the latter is stopped after about 30 min the fibrinogen level is not reduced to less than 100 mg and the change in clotting tests is negligible. The euglobulin lysis time is short (1–15 min) which demonstrates that activator has been formed even in the presence of the inhibitors. The casein tests with the euglobulin fraction demonstrate that plasminogen has been depleted and large amounts of activator have been formed. The lysis test on the labelled standard clot shows that the activity is lower than in the control experiment, but the activator is not completely blocked even in whole plasma.

Trasylol in the dose used interferes much more with the fibrinolytic system. The depletion of plasminogen is slower and the residual lytic activity smaller

These experiments show that the simultaneous administration of large doses of streptokinase and moderate doses of synthetic inhibitors (about 10 g εACA or 0.5–1 0 g AMCHA or PAMBA) reduces the plasminemia and prevents the dangerous period of afibrinogenemia. In the concentrations of the inhibitor used in our experiments SK obviously was not displaced from the plasminogen SK complex as it is suggested by the experiments of HEIMBERGER (10) For practical purpose, half the amount of the inhibitors will be sufficient.

Another question however remains to be solved. HERMEYER (11) has demonstrated in cats that thrombi will not be lysed if the animals have received εACA before SK. He suggests that εACA diffuses into the thrombi and is adsorbed there interfering with the action of the activator coming later to the thrombus. Further studies will be necessary to clarify whether human thrombi and clots adsorb εACA or do not.

Summary

The efficiency of fibrinolysis inhibitors has been compared in vitro on preformed ^{125}J-labelled human plasma clots. p-Aminomethylbenzoic acid (PAMBA) is 12 to 14 times as active as ε-Aminocaproic acid (εACA) and 2 times as active as Aminomethyl-cyclohexane-carboxylic acid (AMCHA) on molar basis. Trasylol, natural occurring polypeptide, is 10^4 to 10^6 times more active than the synthetic inhibitors. The same relationship of activities was found in vivo. A single injection of 5.0 g εACA or 0.5 g AMCHA or PAMBA will effectively interrupt SK-induced fibrinolysis without

activity determination, factor X activity determination, silicone clotting times, stearate activated clotting times, continuous paper curtain electrophoresis, paper strip electrophoresis, protein estimation, thromboplastin generation tests (2) using platelet substitute (1).

The serum subjected to continuous paper curtain electrophoresis was obtained from three patients known to be suffering from severe congenital factor IX deficiency. The serum from each patient was studied separately.

Results

The distribution of the known clotting factor activities (excluding the factor IX) in serum fractions from patients suffering from congenital factor IX deficiency was found to be identical with that in normal serum (Fig. 1). Factor IX deficient serum appeared to contain the same two anti-heparin activities as normal serum (Table I). The α-globulin anti-heparin activity which partly overlapped the factor IX activity of normal blood was demonstrated in serum from all three patients. The fast γ-globulin anti

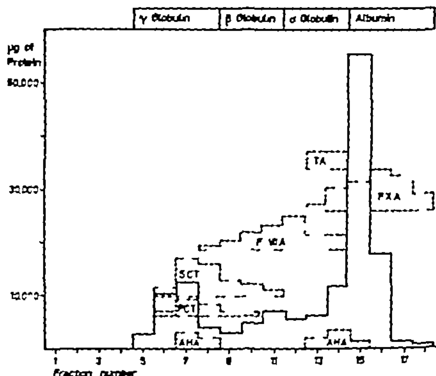


Fig. 1. The relationship between the anti-heparin activity protein fractions and clotting activity in Christmas serum.

Table I

The effect of protein fractions of serum from patients suffering from severe congenital factor IX deficiency upon the heparin retarded clotting time of normal plasma.

Fraction number	Patient A μg protein per ml	Patient A clotting time (min)	Patient B μg protein per ml	Patient B clotting time (min)	Patient C μg protein per ml	Patient C clotting time (min)
1	500	7.30	250	7 15	–	8.45
2	250	7.00	100	6.30	50	8.45
3	750	7.00	100	6.30	1,700	9.00
4	2,750	7 15	50	7.00	3,650	9 15
5	6,050	8.00	2,_00	7 15	4,350	12.30
6	7,000	7.30	10,250	7.30	5,150	32.30
7	6,400	6.15	12 400	4.30	4,300	41.00
8	3,900	6.30	4,100	5.15	4 100	54.00
9	4,950	6.45	2,550	7.30	4,150	13 45
10	4,800	7.30	4,800	7.00	3,200	9.30
11	3,050	7.30	6,900	8.00	4,500	9.15
12	3,500	7.30	5,550	7.30	4,150	9.15
13	4 750	6.15	6,100	5.30	5,350	8.45
14	13,800	5.45	11,500	3.00	9 770	8.15
15	23,000	6.30	33,400	6.30	26,700	7.30
16	21,250	7.00	17,800	9.00	16,350	9 15
17	9 400	7.00	800	7.30	2,000	9.30
18	2,750	7.30	350	7.30	250	8.45
	Thromboplastin generation time using patient A serum 38 sec.		Thromboplastin generation time using patient B serum 27 sec.		Thromboplastin generation time using patient C serum 78 sec.	

heparin activity which has been found to be associated with contact' activation product (3) was demonstrated in two of the three patients. In patient C it was marked by the presence of an anticoagulant which made it impossible to demonstrate the fast γ-globulin antiheparin activity Paper strip electrophoresis of the fractions displaying this anticoagulant activity showed that it was present in the fast γ-globulins (Fig 2)

Further studies on the blood of this patient showed that serum from patient C and fractions of this serum containing fast γ-globulin (fractions 6 7 and 8 in Table I) produced a marked reduction in the thromboplastin generation of normal serum and plasma (Table II) Serum and the fast γ-globulin fractions from patient C also produced a marked prolongation of the recalcification and heparin retarded clotting times of normal plasma. The anticoagulant activity in the serum was thermostable not absorbed by alumina or barium

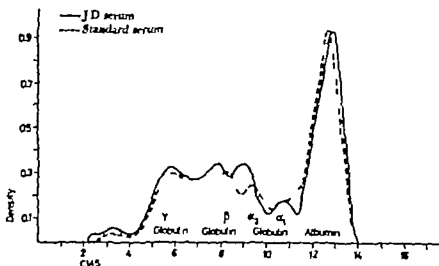


Fig. 2. Comparison of the electrophoretic pattern of JD serum with normal standard serum.

Table II

The effect of serum and protein fractions from patient C upon the thromboplastin generation of normal plasma and serum.

Material added to generation system	Minutes generation 1	2	3	4	5	6
	clotting time of substrate plasma (sec)					
0.1 ml saline	35	12	10	10	10	10
0 1 ml electrophoretic fraction 6						90
bove diluted 1 10						18
0.1 ml lectrophoretic fraction 7	–			–		85
above diluted 1 10						18
0.1 ml lectrophoretic fraction 8					–	86
bove diluted 1 10				–		20
0.1 ml serum diluted 1 10	54	31	24	23	24	20

sulphate and not removed by extraction with ether The anticoagulant was not a heparin-like antithrombin as it did not interfere with the thrombin fibrinogen reaction (Table III)

The anticoagulant was also non-dialyzable, being found in the electrophoretic fractions, subjected to dialysis in the course of desalting and concentration

Paper strip electrophoresis of serum from patient C on visual examination suggested there was a slightly raised level of the fast

Table III

Some properties of serum from patient C.

Effect upon the heparin retarded clotting time of normal plasma		
	clotting time (min)	
0.2 ml saline added	6'00"	5 45
0.2 ml normal serum added	1 45	1 41
0.2 ml Patient C serum	19'00"	22'00"

Effect upon the time taken by 0.1 ml of thrombin solution (7.5 units/ml) to clot 0.2 ml of 1 percent solution of fibrinogen.		
	clotting time (sec)	
0.1 ml saline added	21	19.5
0.1 ml normal serum added	23.5	25
0.1 ml patient C serum added	23.5	24

Effect upon the recalcification time of normal plasma		
	clotting time (min)	
0.2 ml saline added	5'36	5'20"
0.2 ml normal serum added	1'57"	1'53
0.2 ml Patient C serum added	8.35	8.30
0.2 ml Patient C serum incubated at 57 C for 30 min, added	9.55	10.05
0.2 ml Patient C serum, absorbed with 100 mg/ml Barium sulphate for 15 min, added	9.10	9.30
0.2 ml Patient C serum, absorbed with 100 mg/ml alumina for 15 min, added	12 15	12.30
0.2 ml Patient C serum, extracted with ether added	11.00	11 15

γ- and α-globulins. Strips run, stained and scanned under identical condition using identical volumes of serum from patients C and a freeze-dried control serum of known composition confirmed this*

The anticoagulant activity produced a prolongation of the heparin retarded clotting time equivalent to a concentration of 12–15 units per ml of heparin in the serum of the patient.

WEBLER *et al.*(7) have shown that normal serum contains a powerful thrombogenic principle serum thrombotic acceleration (STA) This resembles antiheparin activity in some of its physical

* Hyland Laboratories Albumin 55%, globulin 40%, globulin 11%, globulin 11%, globulin 19%, and total protein 7 g/100 ml (Fig. 3)

properties, thus both are reasonably stable, both are present in factor VII deficient serum and both are absent from serum treated with simple adsorbents, e.g. alumina. Both are also unaffected by coumarin therapy. STA activity could not be equated with factor XI or factor IX but probably represents a combination of these factors (6).

The two different antiheparin activities found by us in normal blood were also found in patients with Christmas disease (factor IX deficiency). This suggests that STA activity when serum products are infused into experimental animals is different from antiheparin activities present in plasma and serum. The results also confirm that factor IX is not likely to be responsible for the antiheparin activity of normal human blood.

Discussion

The present findings provide additional information on the antiheparin activity of blood and confirm that it is not associated with factor IX activity. The anticoagulant activity found in the serum of patient C is similar on physical properties with the circulating anticoagulants reported in the blood of haemophilics, and in cases developing anticoagulants in pregnancy.

The heparin retarded clotting time appeared to detect the circulating anticoagulant and it may be a useful method for showing the early development of anticoagulants.

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Summary

In view of the clinical importance of antiheparin activity in patients with thrombosis and a possible relationship to factor IX (Christmas factor), a study has been made of the clotting activities of protein fractions obtained from cases of congenital factor IX deficiency. The results indicate that anti-heparin activity is not associated with factor IX activity. Further studies were performed on the serum of one patient, in whom a circulating anticoagulant was also found. The heparin retarded clotting time was a sensitive method for the detection of the circulating anticoagulants.

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Preleukaemia

A Report of Four Cases

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Some cases of acute leukaemia are preceded by a variable clinical and haematological syndrome, usually related to a deficiency of at least one cell line in the peripheral blood, the nature of which is obscure until a proliferation of blast cells makes the diagnosis clear. This syndrome has been termed preleukaemia and many of its features were described in a series of 12 cases by BLOCK *et al.* in 1953 (4), and a further series by MEACHAM and WEISBERGER in 1954 (13). A remarkable case lasting 9 years was described by WILLIAMS (19).

The existence of this prodromal syndrome was recorded by GUNZ and HOUGH (9) in their survey of 97 cases of acute leukaemia in New Zealand and accepted by BOGGS *et al.* (5) in their personal study of 322 cases. However, in a further series of 580 cases, ROATH *et al.* (17) were unable to recognise such a phase and no attempt was made to determine its incidence or ascertain its nature. Further authors (18) having accepted this concept, have applied it widely. As well as a so-called myelodysplastic group which would embrace the cases discussed here, they include a variety of disorders which may be complicated by leukaemia, such as aplastic anaemia and the myeloproliferative group of disorders and termed paraleukaemic by HAYHOE (10).

We wish to present 4 further cases to emphasize the existence of this prodromal syndrome and put forward a case for limiting the use of this term to the so-called myelodysplastic group.

Case Reports

Case 1

P.T. 15-year-old school-boy was admitted with 3 weeks history of pyrexial illness with generalised aches and pains which became localised to the lower L thigh. On examination there was soft swelling overlying the L femur with enlarged tender regional lymph nodes. There was no other evidence of lymph node enlargement and the spleen was not palpable. The swelling was surgically explored and large parosteal abscess was drained of pus which on culture yielded heavy growth of staphylococcus aureus. Histology of the abscess wall showed inflammatory granulation tissue only. H was treated with penicillin and streptomycin and made good recovery.

Haematologically (Table I) there was pancytopenia with persistently raised reticulocyte count. Marrow (Table II) showed erythroid hyperplasia with maturation arrest at the metamyelocyte level in the granulocyte series. Megakaryocytes appeared normal. Other tests included serum bilirubin 0.2 mg 100 ml, urinary urobilinogen negative, saline osmotic fragility normal, direct antiglobulin test negative. No precise haematological diagnosis was possible at this stage.

Four months after the original presentation, however, he was re-admitted to hospital following severe nose-bleed. H had extensive purpura, generalised lymph node enlargement and splenomegaly 1 cm below the costal margin. The final haematological picture was that of marked pancytopenia. Hb. 9.4 g/100 ml, platelet count $84 \times 10^{3}/mm^{3}$ W.B.C. $1.5 \times 10^{3}/mm^{3}$ neutrophil polymorphs 3%, lymphocytes 97%. After prolonged search of the peripheral blood film, few lymphoblasts could be found but the marrow showed extensive replacement by sheets of lymphoblasts. A diagnosis of 'aleukaemic lymphoblastic leukaemia' was concluded. H was treated with prednisone 60 mg daily but after short clinical improvement died of overwhelming staphylococcal broncho-pneumonia.

Case 2

S.R.K. retired man of 75 was admitted to hospital following the third episode of epistaxis in 3 months. There was past history of myocardial infarction 5 years previously and recent anginal pain. His main symptom for 3 months before admission had been lassitude. The findings on examination were those of sub-conjunctival haemorrhages and sparse purpuric spots. Regional lymph nodes were not enlarged and the liver and spleen were not palpable.

Haematologically (Table I) there was pancytopenia with consistently raised reticulocyte count which varied between 15–24%. Sternal marrow (Table II) showed marked erythroid hyperplasia granulopoiesis was morphologically normal but the majority of megakaryocytes, which were present in normal numbers, showed little evidence of platelet production. The direct antiglobulin test was negative no L.E. cells were found, serum bilirubin was 0.8 mg/100 ml, blood urea was 116 mg/100 ml, serum proteins and liver function tests showed no abnormality. No haematological diagnosis was reached at this stage when the patient had an attack of left ventricular failure and died.

Autopsy report. Death was due to pulmonary oedema secondary to myocardial ischaemic fibrosis. Spleen, liver and lymph nodes were normal. However both kidneys showed identical changes the capsules were diffusely thickened, green in colour but stripping easily revealing chloromatous kidneys with granular green surface and scattered petechial haemorrhages. On section the green colour was confined to the cortices. Sternal and vertebral marrow appeared hyperplastic with red marrow extending two-thirds of the way down the shaft. *Histological findings.* The kidneys showed gross infiltration of the cortices by sheets of cells, the predominant cell type having

Table I

Preleukaemic peripheral blood findings.

Case	Hb. g/100 ml	H'crit. %	Retics. %	Plat. 10^3/mm³	Total W.B.C. $\times 10^3$/mm³	Diff. W.B.C. % N	E	B	L	M	Film appearance	L.A.P.
1	8.2		7.0	160	2.4	49			44	7	R.B.C. hypochromasia polychromasia	
2	7.2		24.0	89	2.7	65			34	1	R.B.C.: polychromasia N.R.B.C.: 2/100 W.B.C.	–
3	9.6	30	2.0	normal on film	3.6	36			58	6	R.B.C.: anisocytosis. Hypersegmented neutrophils	50
4	7.6	24	3.0	normal on film	3.3	73			25	2	R.B.C. hypochromasia. Pelger Huet anomaly	3

Abbreviations. Hb Haemoglobin, *H'crit.* Haematocrit, *Retics.* Reticulocyte Count, *Plat.* Platelet Count, *R.B.C.* Red Blood Cells, *N.R.B.C.* Nucleated Red Blood Cells, *W.B.C.* White Blood Cells, *Diff. W.B.C.* Differential White Blood Cells, *N.* Neutrophils, *E.* Eosinophils, *B.* Basophils, *L.* Lymphocytes, *M.* Monocytes, *L.A.P.* Leucocyte Alkaline Phosphatase (score)

Table II

Preleukaemic marrow appearances.

Case	Principal feature	M.E. ratio	Percent blast cells
1	Erythroid hyperplasia	1 2	1.5
2	Erythroid hyperplasia	1.5 1	1.0
3	Hyperplasia	5 1	2.0
4	*Granulocytic hyperplasia*	*20 1*	*1.0*

Abbreviation. M.E. ratio Myeloid Erythroid ratio (normal 2.5–15 1)

pale-staining and slightly indented nucleus in keeping with that of myeloblast. Similar cells were found to be involving an abdominal lymph node. Femoral marrow showed predominantly blast cell infiltration with foci of erythroblastic hyperplasia. Liver and spleen were histologically normal and not ffected by the leukaemic process.

Case 3

H.A. a 62-year-old farmer was admitted with 6 months history of pallor shortness of breath, cough, anorexia, loss of weight and 3 episodes of deep ein thrombosis. On examination there was moderate pallor but no other significant features, in particular no enlarged lymph nodes and no splenomegaly

Haematologically (T ble 1) there was normocytic and normochromic anaemia with neutropenia. Large hypersegmented neutrophils were present and megaloblastic anaemia was suspected. Sternal marrow however showed normoblastic erythropoiesis with marked granulocytic hyperplasia serum B_{12} and urinary excretion of FIGLU after histidine load were normal. No haematological diagnosis was possible at this stage He subsequently developed persistent pyrexia 100° to 101 F and an E.S.R. of over 100 mm in the first hour Many investigations were carried out to elucidate the cause of the pyrexia. Repeated blood, marrow sputum and urine cultures were performed, but all were negative including guinea pig inoculation. Many agglutination titres were performed without significant result. Bronchoscopy cystoscopy with retrograde pyelogram were negative. A muscle biopsy was normal, repeated L.E. cell tests were negative until finally laparotomy was carried out. This too proved completely negative and liver biopsy taken the time was histologically normal.

The pyrexia persisted and the patient remained in the same clinical condition. A course of prednisone was given on the assumption tha this was collagen disease and much symptomatic relief was obtained but an abscess developed in the laparotomy wound. Refractory anaemia persisted and repeated transfusion was needed. It was accompanied by neutropenia which varied only slightly in response to steroids or pyogenic infection.

Repeated marrows were performed and evealed increasing granulocytic hyperplasia (Fig 1) chromosome analysis of adequately spread mitotic cells, though small in number failed to reveal aneuploidy of the C group chromosomes or the presence of the Philadelphia chromosome. The alkaline phosphatase score of the leucocytes was not diagnostically significant, but insidiously the percentage of blast cells and promyelocytes began to climb in the marrow (Fig. 1) and 7 months after the initial presentation blast cells began to appear in the peripheral blood. He died 8 months later never attaining remission

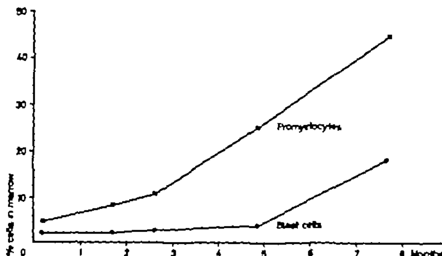


Fig 1 Serial marrow counts in case 3.

Case 4

J D T 56-year-old lerk was admitted in J ly 1965 with history of shortness of breath for 6 months and several hours precordial pain, the nature of which was not discovered. H had lived for 30 years i West Africa where he contracted malaria and filariasis. There was no recent exposure to drugs or chemicals. On examination the only physical sign was that of moderat pallor; there was no lymphadenopathy and no splenomegaly *Haematologically* (T ble I) the conspicuous features were those of a normocytic and normochromic anaemia and leucopenia associated with granulocytic hyperplasia of the bone-marrow The neutrophils present, however showed changes in nuclear morphology Bi-lobed 'pince-nez and single-lobed rounded nuclei with condensed nuclear chromatin pattern were present. They were characteristic of the Pelger Huet nomaly The detailed differential leucocyt count showed single-lobed Pelger-Huet cells, 26%; bi-lobed Pelger cells, 33% polymorphs with normal morphology 12%; lymphocytes, 25%; monocytes, 2%.

N *family study was possible but over the next 2 months the differential count* changed so that nearly all the neutrophils were single-lobed and normal polymorphonuclear neutrophils bsent. As this changing picture pointed to secondary acquired, rather than a primary hereditary cause many other investigations wer performed in an ttempt to find primary cause such as malignant lymphoma or carcinoma but all were negative I particular liver function tests nd serum protein were normal liver biopsy showed no bnormality no parasites were found on examination of blood and stools.

A further diagnosis of preleukaemia was considered and further investigations were performed long these lines. The leucocyt alkaline phosphatase reaction was negative in the bnormal neutrophils. Cytogenetic studies were performed on direct preparations from the marrow but showed no evidence of aneuploidy of the C group chromosomes and the 'Philadelphia chromosome could not be identified. Repeated bone-marrow examinations showed progressive granulocytic hyperplasia with shift to the left which was emphasized by the similarity of myelocytes and single-lobed mature neutrophils. Finally 6 months after the original presentation, he was admitted with bronchopneumonia. The marrow t this time showed 12 % blast cells and 25 % promyelocytes which demonstrated heavy basophilic granulation nd Auer rods. H failed to respond to antibiotics and died shortly fterwards.

Discussion

Clinically the cases showed neither splenomegaly nor lymph adenopathy Symptoms were referrable to the cells deficient in the peripheral blood but all cases were particularly susceptible to infection Haematologically in all cases there was a deficiency of at least one cell line in the peripheral blood and hyperplasia of the corresponding precursors in the bone-marrow

Cases 1 and 2 showed an anaemia with a raised reticulocyte count and erythroid hyperplasia on bone marrow examination normoblasts were present in the peripheral blood of case 2. Reticulocytosis with or without normoblastosis was a feature at some stage in all of the 12 cases of preleukaemia published by Block *et al* (4) and is undoubtedly common in this disorder There was no evidence that this was a regenerative reaction to haemorrhage or haemolysis in case 1 and in case 2 the reticulocytosis was out of all proportion to the severity of the nose bleed Since localised deposits of leukaemic tissue were demonstrated at autopsy in case 2 in which death occurred during the preleukaemic phase it may be postulated that this is a reaction to marrow replacement.

The outstanding abnormalities in cases 3 and 4 were those of persistent neutropenia with abnormal leucocyte morphology and granulocytic hyperplasia of the bone marrow

Marked hypersegmentation of the polymorphonuclear neutrophils was present in case 3 but no evidence of B_{12} or folic acid deficiency could be found. Hypersegmented neutrophils are frequently seen in myeloproliferative disorders but is is not possible to ascertain whether these are of leukaemic origin or reactive. In this case the alkaline phosphatase reaction was normal and cytogenetic studies were inconclusive.

In contrast to case 3 some of the neutrophils of case 4 were single or bi lobed and characteristic of the Pelger Huët anomaly This is a heritable disorder but can be acquired secondary to other disorders particularly acute myeloid leukaemia (8) Dartz *et al* (7) demonstrated that in a case of acquired Pelger Huët anomaly secondary to myeloid leukaemia the proportion of anomalous cells increased as the disease progressed and this pattern was repeated here For this reason acute myeloid leukaemia was suspected from the first but without a demonstrable increase in blast cells the diagnosis could not be made. This emphasizes the fact once more that

there are no established biochemical, cytochemical or cytogenetic features of acute leukaemic cells which unequivocally establishes their nature. The anomalous cells of this case had a negative alkaline phosphatase reaction and direct preparations of bone marrow revealed a normal karyotype.

These 4 cases do not fit into any established haematological diagnosis or classification at their presentation other than preleukaemia which could only be applied in retrospect when a proliferation of blast cells made the diagnosis clear

ROWLEY *et al* (18) have classified preleukaemia into 3 groups, aplastic, myelodysplastic and myeloproliferative and the present 4 cases would seem to fit the myelodysplastic group The inclusion of aplastic anaemia and myeloproliferative disorders within the meaning of preleukaemia is debatable however and we believe a distinction should be drawn between preleukaemic and potentially leukaemic conditions and that aplastic and myeloproliferative groups belong to the latter

Acute leukaemic transformation in aplastic anaemia is only sporadic in published series ADAMS (1) recorded 1 in 27 cases and MOHLER and LEAVELL (15) 1 in a further 50 cases. Moreover in the 45 cases of ISRAËLS and WILKINSON (11) the 60 cases of LEWIS (12) and the *102* cases of BERNARD and NAJEAN (2) this change was not seen to occur

Again in the myeloproliferative group of disorders only a small proportion undergo leukaemic transformation and this *has* been related to radio-phosphorus treatment in polycythaemia rubra vera (14)

A small number of cases of sideroblastic anaemia may undergo leukaemic transformation (5) Although the true nature of this disorder is not known it is usually considered to be a primary disorder which may be complicated by acute leukaemia or a chronic myeloproliferative disorder *changing into a more acute form (6) rather* than preleukaemia. None of the present cases showed abnormal iron staining of the red cell precursors.

Having excluded the aplastic and myeloproliferative groups we should like to confine the term preleukaemia to cases in which there is unexplained anaemia neutropenia or thrombocytopenia or combination of these with a hyperplastic marrow and with no clinical evidence of enlargement of spleen or lymph-nodes, ultimately merging with the full picture of acute leukaemia. The true

nature of preleukaemia so defined is not known but it probably represents the manifestations of an initially occult, slow growing acute leukaemic proliferation, which has been termed smoldering leukaemia by RHEINGOLD *et al* (16) This was revealed at autopsy in case 2 and the abnormal leucocytes in cases 3 and 4 were probably those of a well differentiated slow growing leukaemic process. But no unequivocal method of distinguishing leukaemic from normal leucocytes exists and until this is found, preleukaemia will remain a difficult diagnostic and prognostic problem

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Summary

Four cases of preleukaemia were studied from onset to death. They presented with deficiency of at least one of the cellular components of the peripheral blood, hyperplastic marrow and no enlargement of spleen or lymph nodes. Extensive investigations were not diagnostic until a terminal stage was reached. The nature of preleukaemia is discussed and it is suggested that it is the manifestation of a slow growing occult, leukaemic process rather than a definite haematological condition with a sporadic proclivity to leukaemic transformation.

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Chromosomal Study in Megaloblastic Anaemia of Children

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Occasionally reports have been presented with numerical and/or morphologic chromosomal aberrations in megaloblastic anaemia of adults (2, 3, 7, 9–11, 13, 14). These are characterized by aneuploidy, namely hypodiploidy and the presence of chromatid breaks and gaps, acentric fragments, dicentric or giant chromosomes (2, 3, 10, 11, 13, 14).

In the available literature we were unable to find any cytogenetic data in megaloblastic anaemia of children. Two cases of megaloblastic anaemia in childhood incited us to carry out a cytogenetic investigation. Both were siblings 4 and 3 years old, respectively.

Method

Direct bone-marrow chromosome studies were performed in both patients according to the method previously described (12). The combined Denver (1) and Paris (15) system of nomenclature was used.

Case Reports

The type of megaloblastic anaemia has not been determined accurately. The first patient responded promptly to parenteral vitamin B_{12} therapy; his sister responded to oral folic acid treatment.

The clinical and laboratory data from both patients are summarized in Table I. The asterisks indicate the dates on which cytogenetic studies were performed.

In the first patient, anaemia was present since the age of 4 months; it was diagnosed as being megaloblastic at the age of 18 months, and was attributed to folate deficiency. Therapy with folic acid orally resulted in partial recovery only. Subsequent treatment with parenteral vitamin B_{12}, however, produced an excellent response (Fig. 1).

His sister was anaemic also since the age of 4 months. Folate deficiency was diagnosed at the age of 3 years. Treatment with folic acid by mouth resulted in complete remission.



Fig. 1. Bone-marrow smears from the first patient studied both in relapse (left) and in remission (right). Note the abnormal white cell morphology and the megaloblastic erythropoiesis (left) and the normoblastic erythropoiesis and the normal white cell morphology (right).

Results

The cytogenetic studies revealed the presence of both numerical and structural chromosome aberrations in the two siblings studied in relapse. During remission, however, while on vitamin B_{12} therapy (the one (brother) and folic acid treatment the other (sister)) these changes could not be detected any more.

The results of chromosomal studies from both siblings with megaloblastic anaemia are shown in Table II. In the first patient, in relapse, pronounced hypodiploidy with random loss of chromosomes was present in 8/25 cells. Morphological aberrations were present in 19/25 cells examined. In remission, the chromosome counts reverted to normal, moreover no structural anomalies were found (Fig. 2).

In the second patient (sister of the previous one) during relapse, pronounced hypodiploidy with random loss of chromosomes, was present in 11/32 cells. Morphologic aberrations were present in 24/32 cells examined. In remission, the chromosome counts were normal, and no structural aberrations could be detected (Fig. 3).

Table I

Clinical and laboratory data on the two cases with megaloblastic anaemia of childhood.

Case No. and sex	Date	Clinical status	Age years	Hb. g/100 ml	Reticulocytes	Macrocytosis	Bone marrow megaloblastic changes	Clinical data and response to therapy
1 M	May 1963	relapse	1 1/2	7.6	0.5	+++	+++	anaemia since 4 months of age
	Jan. 1966	relapse	4 2/12	4.1	0.5	++++	++++	diagnosis of M.A. at the age of 18 months
	Feb. 1966	remission	4 3/12	9.0	3.0	0	0	treatment folic acid 5 mg/tid per os
	Apr 1966	remission	4 5/12	11.7	0.2	0	0	partial response
	Jul 1966	remission	4 8/12	11.5	2.0	0	0	*haml* B_{12} 20 gamma/kg B.W./
	Aug. 1966	remission	4 9/12	13.0	0.5	0	0	day I.M.: excellent response
2 F	Apr 1966	relapse	3	9.0	0.5	++	+++	anaemia since 4 months of age
	Jul. 1966	remission	3 3/12	12.0	2.2	0	0	diagnosis of M.A. at the age of 3 years
	Aug. 1966	remission	3 4/12	11.0	0.4	0	0	treatment folic acid 5 mg/tid per os: excellent response

Cytogenetic studies performed

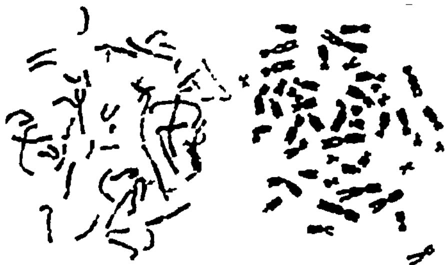


Fig. 2. Bone-marrow metaphase plates from case 1 both in relapse (left) and in remission (right) containing 46 chromosomes each. The arrows point to chromatid breaks and gaps. In remission the chromosomes are of normal size and appearance. Both pictures were taken under the same magnification.

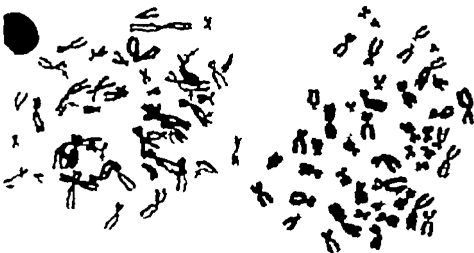


Fig. 3. Bone-marrow metaphase plates from case 2 both in relapse (left) and in remission (right). The left cell contains 47 chromosomes (one extra in the D_{13-15} series), the right one contains 46 chromosomes. The arrows point to numerous structural aberrations (breaks, gaps and an isogap (curved arrow)). 'Giant' chromosomes are also noted. In remission the chromosomes are normal. Both pictures were taken under the same magnification.

Table II

Analysis of chromosomal distribution in two cases of megaloblastic anaemia of childhood.

Case No.	Date	Clinical status	Total No. of cells counted	Chromosome number -40	40	41	42	43	44	45	46	47	92
1	27 1-66	relapse	25	2				2	2	2	17		
	26-2-66	remission	6								4		2
	30-4-66	remission	14			1					12		1
2.	30-4-66	relapse	32	4		3			3	1	20	1	
	19-8-66	remission	14		1						12		1

Discussion

FORD *et al.* (7) reported a case of pernicious anaemia with pronounced hypodiploidy but subsequently these findings were attributed to technical imperfections (8). COURT BROWN *et al.* (5) and DE LA CHAPELLE and GRÄSBECK (6) in studies of 5 and 4 cases, respectively failed to find significant abnormalities.

On the other hand ASTALDI *et al.* (2, 3) portrayed numerical and morphologic chromosome changes in a woman with untreated pernicious anaemia. The abnormalities were apparently less pronounced after vitamin B_{12} therapy (4). Similar results were reported by FORTEZA and BÁGUENA (9) in another case of pernicious anaemia. MAC DIARMID (14) reported an increased incidence of aneuploidy and alterations in individual chromosomes in 5 patients with pernicious anaemia.

In a recent study of three patients with pernicious anaemia reported by KIOSSOGLOU *et al.* (13) numerical and structural aberrations of chromosomes were present. These were characterized by pronounced hypodiploidy and the presence of chromatid breaks and gaps acentric fragments and dicentric or giant chromosomes. After vitamin B_{12} therapy the morphologic aberrations were greatly reduced. The numerical anomalies persisted, although significantly decreased. POWSNER and BERMAN (16) found abnormally long and thin chromosomes in their cases with no other numerical or structural abnormalities. Recently HEATH (10) reported on 14 cases of anaemia associated with deficiency in vitamin B_{12} and/or folate. In 7 definite structural changes were present consisting in increased chromatid breaks, incomplete contractions (the equivalent, perhaps, of what we have termed giant chromosomes) and cen-

tromere spreading. These anomalies were no longer present after appropriate treatment.

In the present study two cases of megaloblastic anaemia of childhood were investigated. Both patients were siblings and were studied both in relapse and in remission. In the two siblings (brother and sister) numerical and structural chromosome aberrations were found, which reverted to normal after vitamin B_{12} therapy in one and folic acid treatment in the other.

The cytogenetic abnormalities found in megaloblastic anaemia of adults and children may be related to the metabolic role of vitamin B_{12} and folate. It is well known that both are involved in the metabolism of the DNA. Deficiency of either of these vitamins may therefore lead to disordered DNA synthesis which may be responsible for morphologic aberrations, such as chromatid breaks and gaps, acentric fragments, dicentric or giant chromosomes.

Summary

Cytogenetic studies were performed on two children (brother and sister) with megaloblastic anaemia. In both siblings chromosomal aberrations were found during relapse. They were no longer present while the patients were in remission on vitamin B_{12} and folate replacement.

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Hageman Factor Deficiency

Report of a Case Found in Japanese Girl

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K. Teramura and Y. Sunaga

Hageman factor deficiency is a rare disorder which affects both sexes and is characterized by prolonged clotting time of venous blood without abnormal bleeding tendency. The disorder is usually discovered by chance.

This is to report a case with this disorder found in Japanese, whose abnormality was detected accidentally during routine pre-operative laboratory studies.

Report of Case

Y.N., a 5½-year-old Japanese girl was admitted to the Toranomon Hospital in September 1966 for tonsillectomy when she was found to have prolonged clotting time during routine pre-operative studies. She had adenoidectomy at 3 years of age without unusual bleeding. She is normally active and has never been considered by her parents as being more susceptible to bruises than her brother and other children. She had never had swollen joints, large ecchymoses, or prolonged bleeding following cuts. Family history revealed that the parents of the propositus are first cousins. The family tree is shown in Fig. 1. None of the members in the family is said to have hemorrhagic diathesis.

The physical examination was entirely negative except for enlarged tonsils and hard of hearing due to adenoids.

Routine laboratory studies: Urinalysis negative; hemoglobin 12.1 g %; red blood cell count $4.2 \times 10^6/mm^3$; white blood cell count $10{,}000/mm^3$ with normal differential count; platelet count $32 \times 10^4/mm^3$; bleeding time (Duke) 2 min; blood clotting time (Lee White, normal 5 to 13 min) 31 min; plasma prothrombin time (Quick) 62%; serologic test for syphilis negative; blood groups B, Rh-positive.

Study of the Clotting Defect

The results of routine clotting studies of patient's blood are shown in Table I. As can be seen from the table, the patient's

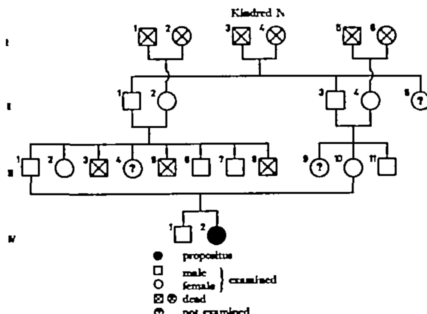


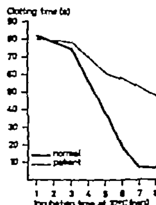
Fig 1 Hageman factor deficiency kindred.

Table I

Results of investigation of the hemostatic mechanism in patient Y N.

Bleeding time (normal 1 to 3 min)	2 min
Capillary resistance (Rumpel-Leede)	normal
Platelet count (normal 15–39 × 10^4)	31.6×10^4
Platelet morphology	normal
Clotting time (Lee White normal 5 to 15 min)	29 31 36 min
Silicone clotting time (normal 23 to 28 min)	34 42 min
Recalcification time (normal 100–205)	24 min
Plasma prothrombin time (Quick, one stage; normal > 70%)	62%
Prothrombin consumption (normal > 31)	11
Clot retraction	normal
Fibrinogen (FI-test)	normal (over 100 mg/dl)

blood exhibited greatly prolonged clotting time and recalcification time, slightly prolonged plasma prothrombin time, and abnormal prothrombin consumption. From these results, a disturbance of blood thromboplastin formation seemed most likely and further investigations on factors concerning this phase of clotting were made. As the propositus is a girl, it seemed unlikely that she had either AHF deficiency or PTC deficiency and it seemed more



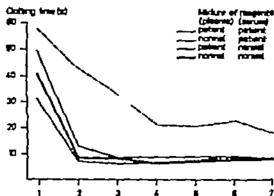


Fig 2 *Fig 3*

Fig.2. Result of thromboplastin-generation screening test.
Fig.3. Result of thromboplastin generation test.

likely that she had either PTA deficiency or Hageman factor deficiency

Thromboplastin-generation screening test showed marked abnormality as shown in Fig 2. The results of thromboplastin generation test revealed that both the normal serum and the normal $BaSO_4$ absorbed plasma corrected the abnormal thromboplastin generation of the patient's blood (Fig 3)

Recalcification time using untreated tube was markedly prolonged (24 min) When the test tube sensitized by rinsing normal diluted serum (one part of plasma in 100 of isotonic saline) followed by washing six times with isotonic saline was used (1) recalcification time of the patient's plasma was almost normalized (3 min 24 s)

Citrated lyophilized plasma was prepared using silicon coated glassware, and was sent to Dr O D RATNOFF of Western Reserve University Cleveland, Ohio, and to Dr J P SOULIER of Centre Nationale Transfusion Sanguine, Paris, by air mail, who very kindly performed specific assay of PTA and Hageman factor The results showed that whereas PTA activity of the patient's plasma was over 100% Hageman factor activity was less than 1%

Family Study

Eleven out of 14 living relatives of the propositus were available for routine coagulation study as shown in Fig 1 Hemoglobin con-

centration, red blood cell count, white blood cell count, differential count, platelet count, bleeding time, blood clotting time (Lee White) recalcification time, one stage prothrombin time (Quick) and tourniquet test were performed on these members of the family None of them showed definite abnormality The results of coagulation time and recalcification time are shown in Table II

Citrated lyophilized plasmas from parents, both paternal and maternal grandparents were prepared, using siliconized glassware, and again sent to Dr O D Ratnoff for Hageman factor assay The results obtained in his laboratory are shown in Fig 4 The father paternal grandfather and maternal grandfather showed low Hageman factor activity whereas that of the mother maternal grandmother and paternal grandmother were within normal range.

Discussion

Hageman factor deficiency is quite unique among coagulation factor deficiencies in that these individuals ordinarily do not show abnormal hemorrhagic symptoms even after trauma, surgical procedures, although blood clotting time is markedly prolonged as seen in severe hemophilia The case reported here is not an exceptional one in this respect.

Table II

Results of clotting time and recalcification time in members of kindred N.

			Age	Clotting time (Lee-White) (normal 5 to 15 min)	Recalcification time (normal 100 to 200 s)	Comment
II	1	Paternal grandfather	70	10 ½	191	
	2	Paternal grandmother	64	10	151	
	3	Maternal grandfather	79	9 ½	110	
	4	Maternal grandmother	69	9 ½	91	
III	1	Father	41	8		Thromboplastin generation test normal
	2	Paternal aunt	39	10 ½		Thromboplastin generation test normal
	6	Paternal uncle	29	11	140	
	7	Paternal uncle	25	10	130	
	10	Mother	33	12	168	
	11	Maternal uncle	29	9 ½	122	
IV	1	Brother	7	10	151	

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A Survey for Erythrocyte Glucose-6-Phosphate Dehydrogenase Deficiency in Rumania

J. H. Schneer

Hemolytic disorders following consumption of certain vegetables and drugs have long been known, but only 12 years ago it was recognized that the deficiency of erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) is in the background of these disorders [2]. The G-6-PD deficiency is prevalent amongst some ethnic groups including American and Africans Negroes, Caucasians of Mediterranean countries and some South-east Asian populations [6, 8, 9, 12]. In Central and Northern Europe, the G-6-PD deficiency appears too, but only sporadically [3, 4, 5, 13].

In Rumania, the first case of G-6-PD deficiency – a chronic non-spherocytic anemia – was observed in 1964 by Kovacs *et al.* [7]. One year later, we reported two cases of favism and the investigation of the relatives revealed other three defective subjects [10]. The absence of previous data regarding the incidence of this enzymopathy in our country prompted us to conduct a search for G-6-PD deficiency among a Rumanian population and in 1966 we exposed the preliminary results [11].

The purpose of this paper is to report the actual data of our survey.

Materials and Methods

Venous blood specimens were obtained from randomly selected patients and normal individuals, from the Department of Paediatrics and the Policlinic of Huşi (Rumania).

The qualitative G-6-PD activity was determined on freshly-drawn heparinized blood, by the methemoglobin reduction test of Brewer *et al.* [1]. Subsequently, the deficient individuals and their relatives were investigated clinically, hematologically and genetically

Table. G-6-PD deficient individuals

No.	Subject	Years	Sex	Pedigree	Diagnosis	Methemoglobin reduction test
1	G. O	3	M	propositus	favism	complete deficiency
2	G. R.	25	F	mother	healthy	partial deficiency
3	G. V	28	F	aunt	healthy	partial deficiency
4	C. A.	2	M	cousin	healthy	complete deficiency
5	M. D	1	M	propositus	favism	complete deficiency
6	M. C.	29	F	mother	healthy	partial deficiency
7	B. C.	49	F	grandmother	healthy	complete deficiency
8	B. V	11	M	uncle	healthy	complete deficiency
9	G. C.	15	M	propositus	hemophilia A, acut hemolytic anemia	complete deficiency
10	G. L	37	F	mother	healthy	complete deficiency
11	S. \	17	M	propositus	healthy	complete deficiency
12	S. E.	47	F	mother	healthy	partial deficiency
13	S. R.	9	M	brother	healthy	complete deficiency
14	S. F	7	M	brother	healthy	complete deficiency
15	D. A.	64	F	grandmother	healthy	complete deficiency
16	B. E.	37	F	aunt	healthy	partial deficiency
17	A. A.	0.5	F	proposita	acute hemolytic anemia	complete deficiency
18	A. L.	43	M	father	healthy	complete deficiency
19	L. G.	66	M	propositus	thalassemia minor	complete deficiency
20	H. M.	27	F	daughter	healthy	complete deficiency
21	D. A.	31	F	daughter	healthy	complete deficiency
22	D. V	4	M	grandson	adactylia of the right hand	complete deficiency
23	G. A.	31	F	proposita	history of favism	complete deficiency
24	G. E.	73	F	mother	chronic anemia	complete deficiency
25	G. D.	81	M	father	healthy	complete deficiency
26	G. E.	13	F	daughter	healthy	partial deficiency
27	G. C.	9	M	son	healthy	complete deficiency
28	O. G.	3	M	propositus	favism	complete deficiency
29	O. A.	24	F	mother	healthy	complete deficiency
30	D. C.	68	M	grandfather	healthy	complete deficiency
31	M. T	35	F	aunt	healthy	complete deficiency
32	M. P	6	M	cousin	healthy	complete deficiency

In this study the screening test employed, did not detect as propositus, partially G-6-PD deficient females.

Results

The methemoglobin reduction test was performed on 890 males and 792 females. Complete G-6-PD deficiency was found in 8 probands

and the survey of 94 relatives revealed other 24 deficient individuals. All the deficient subjects are of Rumanian origin between their families are no relation. The clinical and hematological data for these 32 *deficient individuals are listed in the table.*

Discussions

This is the first attempt at estimating the incidence of G-6-PD deficiency in a Rumanian population, randomly selected. Considering only the test performed on males, the G-6-PD deficiency appears to attain 1 8% (gene frequency 0.0067–0 018)

It is difficult to explain the appearence of the enzymopathy Since in our country malaria was endemic in previous years, the G-6-PD deficiency could be explained by the controversial malaria theory Another possibility is that the genopathy was brought by the mediterranean peoples who colonized the ancient territory of Rumania (the ancient Dacia)

We suppose that the G-6-PD deficiency is not restricted to the area investigated in our survey and we think that it would be advisable to continue such studies, not only for the genetic and anthropological significance of this estimation but also for the prevention of the hemolytic states in defective subjects.

Summary

A survey for erythrocyte glucose-6-phosphat dehydrogenase deficiency was carried out amongst 1682 randomly selected Rumanian individuals, by the methemoglobin reduction test. 32 individuals were found to have G-6-PD deficiency Considering only the G-6-PD deficient males, the enzymopathy appears to attain 1.8% (gene frequency 0.0067–0.018)

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Acid Phosphatases of Platelets during Coagulation and Storage of Blood

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Platelet factor 3 (PF3) belongs to the basic properties of platelets by which the haemostatic function of these blood elements is realized. It is evident that we can speak about lipoprotein the exact localisation of which in the ultrastructure of platelets is still open [1].

In the previous work it was shown that the acid phosphatases (APh) liberated from the platelets into serum during the coagulation of blood are probably linked to lipoprotein which is responsible for the so called platelet like activity of serum [2].

In this work we tried to find the basic correlation between activity of APh and PF3. From the point of view of lysosomal theory APh can be used as biochemical marker of lipoprotein of lysosomal origin [3].

Material and Methods

Blood was taken from healthy volunteers. The serum for studying PF3 like activity was acquired from native platelet rich plasma (PRP) without use of anticoagulant as described previously as well as the setting of the activity of APh of serum or plasma without the use and with the use of detergent-analogon Triton X 100 [2].

The shortest fibrinogen time (SFT) was set as O'Brien described [4].

APS ("activité plaquettaire du sérum") was carried out according to ALAGILLE and SOULIER [5]. Results are given as differences in prothrombin consumption between 4–6 min after recalcification of test mixture. The substrate plasma without platelets was prepared for both coagulation tests according to the last mentioned method.

The fresh blood stored in ACD solution was kindly supplied by Transfusion Service Žilina. The blood was stored at 4°C and in certain intervals the samples were taken under sterile conditions. Platelets were counted by the [illegible] method [6]. Plasma was separated from the samples by centrifugation at 4°C at 2,000 × *g* twice, each centrifugation lasting

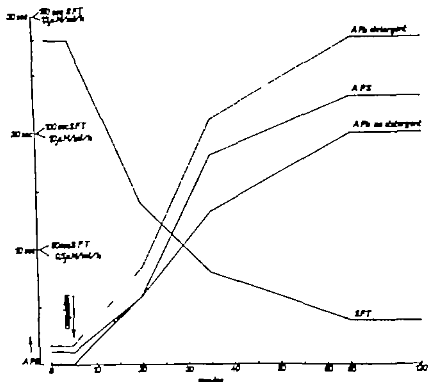


Fig.1 Time dependence of liberation of acid phosphatase (APh) and PF3 like activity into serum from native platelet rich plasma (PRP). *SFT* = shortest fibrinogen time *APS* = platelet activity of serum.

30 min. No formed elements could be observed in this plasma under the phase microscope. In the plasma we observed the haemoglobin content [7], the activity of APh without and with detergent.

The thromboplastic activity by means of methods mentioned above [4 5] with modification forced by using of citrate plasma instead of serum as tested material. The basic mixture which was the same for both methods contained 0.3 ml of platelet poor substrate plasma and 0.2 ml of tested plasma. T recalcification 0.5 ml of 0.025 M $CaCl_2$ were used.

Haemolysate was prepared by dilution of isolated erythrocytes by destilled water to desired concentration of haemoglobin [8].

Results

The time dependence of liberation of APh and PF3 activities into serum is given in figure 1 The curves show the correlation between both activities. The activities in serum seem to depend on the retrac

Table II. Changes in some activities in plasma from blood stored in ACD solution at 4°C

Blood No.	Day	Thrombocytes	Haemoglobin mg%	Acid phosphatase µM/ml/h no deterg.	Acid phosphatase µM/ml/h with deterg.	SFT sec	APP sec
	0	165 000	10	0.140	0.216	68	0
1	7	132 000	20	0.224	0.714	24	3.7
	14	94 000	66	0.478	0.840	16	12.3
	21	50 000	208	1.204	2.534	15	20
	0	223 000	14	0.250	0.420	52	2
2	7	192 000	14	0.345	0.870	30	8
	14	160 000	47	0.435	0.920	22	10
	21	101 000	71	0.770	1.340	20	20
	0	217 000	28	0.226	0.232	31	0.5
3	7	168 000	48	0.546	1.172	18	18
	14	130 000	126	0.868	1.680	18	23
	21	66 000	181	0.742	1.594	18	23
	0	186 000	16	0.084	0.112	110	0
4	7	130 000	50	0.350	0.560	31	3
	14	101 000	128	0.882	1.338	20	20.2
	21	95 000	198	0.700	0.994	18	14.2
	0	207 000	28	0.126	0.196	66	0
5	7	128 000	48	0.294	1.134	17	10.5
	14	98 000	144	0.588	1.680	17	16.5
	21	48 000	226	1.148	2.072	17	22

HOROWITZ *et al.* [9] shows as for quantitative changes after ultracentrifugation the similar values as the activity of APh. About 15–20% of the activity APh liberated into serum remains also after centrifugation at 20,000×*g* in the supernatant. In such supernatant no tromboplastic activity could be traced by coagulation tests we used.

When following the curves showing the changes of given values in plasma obtained from the stored blood, the time correlation of the appearance of thromboplastic activity concomitantly with the activity of APh is also evident. We assume that we dealed with material of platelet origin according to WOLF [10] who described the platelet dust liberated into plasma during storage of blood. The enormously high values of APh obtained especially by means of detergent indicate *that there must be a rest of the platelet lysosomes.* This should prove electronoptical findings [11]. Even if the older samples of plasma show comparatively high values of haemoglobin, erythrocytes participated in both observed activities only very little. Haemolysate of isolated

erythrocytes the value of which was 200 mg% haemoglobin shows the activity of APh with detergent about 0 1 units of BESSEY LOWRY No thromboplastic activity could be traced by tests used by us.

The fact that during storage of blood, except others, also the rest of platelet lysosomes is liberated into plasma is interesting both from theoretical and from practical points of view If the correlation be tween by APh labeled lipoprotein and PF3 activity is proved by further experiments, it could help to solve the question of PF3 origin. We should also mention the finding of KOWALSKI *et al.* [12] that platelet APh is activated by kaolin when the values approached the values obtained by means of Triton X 100 only On the other side the activity of PF3 was proved by means of the very kaolin [13 14]

The fact that highly active sediment of serum acquired by ultra centrifugation is completely captured in coagulum of native PPP might mean that during retraction of coagulum a part of thrombo-plastic activity liberates into serum and perhaps is to ensure the perfect course of coagulation.

The increased availability of PF3 in plasma of stored blood could probably help to explain the well known clinical experience regarding the unsuitability of transfusion of stored blood to patients suffering from thromboembolic diseases.

Acknowledgement

W are indepted to Mrs. L. KOPÁSKOVÁ for her excellent technical assistance in the conduct of these studies.

Summary

A time correlation of appearance of acid phosphatase and platelet factor 3 activities in the serum obtained from native platelet rich plasma has been found. But none of these activities was liberated from the platelets into plasma or serum close to the time when the coagulum developed in vitro. The liberation of both activities could be observed only after retraction of the coagulum.

Concomitantly with the liberation of acid phosphatases the platelet factor 3 activity in plasma obtained from stored blood raised. The high values of acid phosphatases in plasma give evidence to their platelet lysosomal origin.

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Bone Marrow Injury and Repair

Irradiation and Mechanical Disruption[1]

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Exposure of the femur and tibia of the rat to 4 000 r of x irradiation results in permanent aplasia of the bone marrow [1]. Two months post irradiation, when the aplasia has been well established, autologous marrow removed from an unirradiated bone and implanted in the irradiated area can reconstitute hemopoietic activity [2]. It has been proposed that the limiting factor which determines the existence of aplasia or hemopoiesis is the condition of the microcirculation of the marrow. This sinusoidal structure is evidently uniquely capable of supporting the proliferation of hemopoietic tissue. When it is destroyed by irradiation, or otherwise, the hemopoietic marrow atrophies. The restoration of normal activity in the irradiated marrow cavity by transplantation of tissue is presumably accomplished by adventitial cells capable of proliferating to reconstitute the microcirculation. This experiment was designed to learn if normal marrow from the shielded portion of an irradiated bone could be induced by mechanical mixing to proliferate in the aplastic portion.

Materials and Methods

Radiation was performed on the distal half of the tibia in groups of 10 male Wistar rats of about 400 g. The rats were placed in individual boxes and shielded by layers of lead to protect the body, upper legs and feet. The radiation source was 250 kvp-15 mA with ½ mm

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copper and 1 mm aluminium filter The target-to-skin distance was 35 cm. Dose rate was 250 /min, and exposure time was 15.5 min. The half-value layer was 1.2 mm copper. The total exposure dose was 4 000 The original groups of 10 rats were reduced by 20 % because of autoamputation of irradiated legs. Shielding the feet during irradiation prevented this.

When anesthesia was required, pentobarbital was injected intraperitoneally Mechanical disruption of tibial marrow was accomplished by introducing a 1 ½ in, 21 gauge needle into the marrow cavity at the femorotibial joint. Then stainless steel wire was passed through the needle into the cavity and the marrow was mixed by 20 up-and-down strokes of the wire trochar

The marrow was disrupted at varying intervals after the irradiation (table I). In one group of animals the operation was performed before irradiation and in one group it was performed without irradiation. Only marrow of the left leg was disrupted so that the right could serve as control.

The animals were killed with ether and the tibias were removed and fixed in buffered formalin for 3 days, decalcified in Perenyi' solution for 5 days, imbedded in paraffin, sectioned at 6 µm and stained with hematoxylin and eosin.

Results

Those bones in which the marrow was not mechanically disrupted were examined at intervals up to 8 weeks after irradiation and the typical cycle of changes was observed sinusoidal dilation, intra medullary hemorrhages, hyperplasia and then hypoplasia [1] In all

Table I. Recovery of hemopoiesis in hemiirradiated tibia 4000 r to distal end followed by mechanical disruption of marrow

Group (10 rats each)		Marrow disrupted after irradiation weeks	Interval from disruption of marrow until death, weeks	Interval from irradiation of marrow until death, weeks	Recovery of hemopoiesis
1	A	1	9	10	yes
	B	1	13	14	yes
	C	1	15	16	yes
2	A	2	6	8	yes
	B	2	8	10	yes
3	A	4	4	8	yes
	B	4	6	10	yes
	C	4	8	12	no
	D	4	10	14	no
4	A	6	4	10	no
	B	6	6	12	no
	C	6	10	16	no
5	A	8	2	10	no
	B	8	6	14	no

of the irradiated tibias there was a line of demarcation by the 8th week in the distal end of the cavity the hemopoietic marrow was replaced by loose connective tissue, rare lymphocytes and plasma cells and few erythrocytes. The proximal end contained normal marrow

The effect of mechanical disruption of marrow in the unirradiated tibia was studied after the 5th postoperative day Marked endosteal proliferation was seen and in some areas the medullary cavity became completely filled with trabecular bone. Two, 3 and 4 weeks after disruption of the marrow some trabecular bone remained in the cavity but endosteal proliferation subsided with time.

Disruption of marrow preceding irradiation by 5 days did not modify the effects of irradiation. At 8 weeks there was fibrosis in the distal end of the cavity and in the proximal end there was normal hemopoietic marrow plus trabecular bone. The irradiated distal right tibia in every animal became fibrotic and aplastic. Results of the combined injury are shown in table I. When mechanical disruption of the left tibial marrow followed irradiation by one or two weeks (groups 1 and 2) hemopoietic cells were found in the irradiated areas 4 to 15 weeks postoperatively Hemopoietic tissue and trabecular bone were present through the entire length of the cavity In some bones islands of normal tissue were scattered among aplastic areas. When disruption followed irradiation by four weeks (group 3) there was recovery of hemopoiesis in the distal cavity of some animals, but not all. When disruption followed irradiation by 6 or 8 weeks (groups 4 and 5) there was only fatty and connective tissue in the distal end of the cavity 2 to 10 weeks postoperatively

Discussion

When the marrow was mechanically disrupted, the cavity quickly became filled with trabecular bone surrounded by osteoblasts. The endosteum was hypertrophic. In succeeding weeks the hypertrophy subsided. The bony replacement was complete in some bones, patchy in others. Hemopoietic marrow was present in the areas of no bone and in the spaces between the bony trabeculae.

When the marrow was mechanically disrupted after irradiation, the irradiated and unirradiated tissues were tumbled and mixed within the bony cavity The spotty recovery of the marrow in the irradiated portions of the bone indicate that the intent of the procedure was

accomplished. Normal marrow was forced into the aplastic tissue and established itself there. But the presence of aplastic areas in the proximal cavity suggests that irradiated tissue was moved into the shielded areas and it was not invaded or converted by the adjacent normal tissue.

As the irradiation lesion matured it became more fibrotic. After four weeks the efforts to mix the marrow in the two ends of the cavity were unsuccessful, perhaps because the wire trochar only channeled the irradiated tissue and did not replace it.

It is proposed that the growth of hemopoietic marrow in irradiated portions of the tibia occurred because adventitial tissue was displaced into those areas permitting the marrow's microcirculation to be reestablished there. This would indicate that the marrow's adventitia is not aggressive: it does not propagate into irradiated areas by its own initiative. Hemopoietic tissue, on the other hand, seems to develop wherever there is adventitia to support it.

Summary

Post-irradiation aplasia in the marrow of the rat's tibia developed only in the irradiated distal half. When the marrow of the unirradiated half was moved into the irradiated portion by means of mechanical disruption of the bony cavity, hemopoietic marrow developed in the irradiated portion.

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Eosinophils and Dietary Histamine in the Rat

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Eosinophils are most numerous in the gastrointestinal tract and the skin and spend most of their life in these tissues [1]. Their function is still obscure, but Archer [2] considered that they inactivated histamine: this conclusion was based on the occurrence of a local eosinophilia following intradermal injection of histamine, which could be inhibited by injection of an antihistamine immediately after the injection of histamine. This hypothesis would be strengthened if the turnover or number of eosinophils altered in response to an alteration in histamine balance or in response to the continuous administration of antihistamine. In the present work, continuous infusions of tritiated thymidine were used in rats to investigate the effect of histamine-high diet, histamine-low diet and normal diet with antihistamine, on eosinophil turnover and populations. One of the authors has previously studied eosinophils in normal rats by this technique [1].

Material and Methods

Seven (B 1–7) adult male black-hooded PVG/C rats (weight 300 g) and 16 (S 1–16) adult male Sprague-Dawley albino rats (weight 180–210 g) were divided into 4 groups according to diet. Each diet was given for 16 days before the rats were killed. In each group, 2, 3 or 4 rats were given 8 or 6 days continuous intraperitoneal infusion [3] of 1 μc tritiated thymidine (specific activity 3.0 C/mM)/g/day starting 8 or 10 days after the special diets were begun and ending when the animals were killed. The detailed treatments in each group were as follows.

Normal diet. Six control rats (B 1–2 and S 1–4) received a normal cubed 41 B diet [4]. Three were infused with tritiated thymidine for 6 days (B 1 and S 1–2) and one for 8 days (B 2).

Histamine-high diet. Seven rats (B 3–5 and S 5–8) each received 15 g fresh minced raw meat daily. This diet supplies large quantities of exogenous histamine and probably also stimulates the formation of endogenous histamine [5]. Three rats (B 3 and S 5–6) were infused with tritiated thymidine for 6 days.

Histamine-low diet. Six rats (B 6–7 and S 9–12) each received 15 g daily of diet containing less than 0.05 μg histamine/g [6]. One rat was infused with tritiated thymidine for 8 days (B 6) and 2 for 6 days (S 9–10).

Antihistamine. Four rats (S 13–16) on a normal 41 B diet each received 2.5 mg Mepyramine maleate/day. This was given in the drinking water to S 15–16 throughout, and in the drinking water to S 13–14 for 10 days, then in continuous intraperitoneal infusion for 6 days. Rats S 13–14 were infused with tritiated thymidine for 6 days.

After 10 days of the special diets, absolute eosinophil counts [7] were made on blood samples taken from 4 rats in each group (S 1–16). Blood films were taken from the tail veins of each infused rat at intervals during the infusion and fixed in 100% methyl alcohol; marrow smears (fixed in 100% methyl alcohol) and tissue blocks (fixed in formol-saline) were taken at necropsy from each infused rat. Tissue blocks were also taken at necropsy *from rats B 4–5 (histamine-high diet) and B 7 (histamine-low diet). Sections (5 μm)* were prepared from the tissue blocks; those from the infused rats, and their blood films and marrow smears, were dipped in Ilford K.5 nuclear emulsion or covered with Kodak AR 10 stripping film and these autoradiographs were exposed for 4 weeks at 0–4 C. All the sections, films and smears were stained with Mayer' haemalum and Biebrich scarlet to identify the eosinophils [8]. Labelled and unlabelled eosinophil nuclei to total of between 50 and 200 were counted in each film, smear or section. Eosinophils in the marrow were not differentiated into myelocyte, metamyelocyte, stab form or mature eosinophil.

Results

The marrow smears from each infused rat showed that cells with eosinophil granules formed 8–10% of the total cell population and the percentage labelling of these cells was 97–100%.

In the black hooded rats, differential cell counts on the blood films showed that eosinophils formed 5% of the circulating white cells, and between 100 and 120 eosinophils were present in each film. These values are within normal limits [1]. In the Sprague Dawley rats, absolute eosinophil counts on blood samples were between 122 and 288 eosinophils/mm^3 which is within the range given by RYTÖMAA [9] for normal rats weighing 210 g. Thus the total number of blood eosinophils was within the normal range irrespective of diet.

The percentage of eosinophils labelled in each blood film was used to establish their rate of turnover. Sequential entry to and random removal from blood has been established for normal rat eosinophils [1] and thus requires that

$$\log_e \left(\frac{1}{1-f} \right) = kt,$$

where f is the fraction of eosinophils labelled at time t, and k is the number of eosinophils added to the circulation in unit time, expressed as a fraction of the number normally present in the circulation. If log (1/1 – f) is plotted against t and a best fitting line is drawn, the slope of this line gives the value of k. k was determined in this way for each rat, and (1/2k) (the half life of an eosinophil in the circulation) is given in table I. The intercept of the line on the abscissa gives the time at which the eosinophils begin to leave the bone marrow and enter the blood. The time between the start of the infusion and this point is the length of time from the end of deoxyribonucleic acid synthesis of an eosinophil myelocyte to the exit from the marrow of the consequent eosinophil leucocyte (table I) The half life of eosinophils in the blood, and the time of appearance of labelled eosinophils in the blood, do not differ significantly from the normal in any dietary group

In the tissues the distribution of eosinophils was normal in all rats [9] No significant difference was found in either total number or percentage labelling of eosinophils between black-hooded and Sprague Dawley rats, and the values for the two strains of rat have therefore been pooled for the statistical analysis there was also no significant difference between the percentage labelling of eosinophils in stomach, small intestine and large intestine, which have therefore also been

Table I. Half-life of eosinophils in the blood and interval between beginning of tritiated thymidine infusion and appearance of labelled eosinophils in the blood

Rat	Diet	Half-life h	Time of appearance of labelled eosinophils h
B 1 B 2	Normal	8–12	60–73
B 3	Histamine-high	11.5	67½
B 6	Histamine-low	10.1	61
S 1	Normal	10.0	51
S 2		8.8	50
S 5	Histamine-high	8.4	40
S 6		10.0	40
S 9	Histamine-low	5.9	44
S 10		8.0	48
S 13	Normal diet with antihistamine	9.8	46
S 14		8.3	42

Range of normal from Foot [1].

pooled for the analysis. The mean values and the standard deviations for total number and percentage labelling of eosinophils in the tissues are given in tables II and III and comparison using the Student t test showed no significant differences between the dietary groups.

Discussion

CODE [10] suggested that eosinophils contained histamine, but the amount is insignificant when compared to that in mast cells [11].

There is good evidence that the eosinophil is an antagonist to histamine. This property has been demonstrated in eosinophils from rabbits [12], from horses [13, 14], and from man [14]. WELSH and

Table II. Total number of eosinophils in 2 mm^2 of a 5 μm section from each rat tissue: mean and standard deviation for each dietary group

Diet	No. of rats	Spleen	Stomach	Small intestine	Large intestine
Normal	4	23 ± 4	208 ± 68	130 ± 35	145 ± 52
Histamine-high	5	18 ± 3	233 ± 75	132 ± 36	139 ± 21
Histamine-low	4	23 ± 12	204 ± 64	132 ± 24	167 ± 34
Normal diet with antihistamine	2	19 ± 6	164 ± 53	111 ± 38	177 ± 40

Table III. Percentage of eosinophils labelled in rat tissues: mean and standard deviation for each dietary group

Diet	No. of rats	Spleen	Stomach and small and large intestine
6-day tritiated thymidine infusion			
Normal	3	79 ± 10	43 ± 21
Histamine-high	3	70 ± 4	40 ± 14
Histamine-low	2	70 ± 7	37 ± 16
Normal diet with antihistamine	2	74 ± 9	50 ± 19
8-day tritiated thymidine infusion			
Normal	1	88	79 ± 8
Histamine-low	1	83	83 ± 10

GREER [15] produced electron micrographs which demonstrated phagocytosis of mast cell granules by eosinophils in the peritoneal fluid of a rat.

There is also good evidence of a chemotactic effect of histamine on eosinophils in some species but not in others. When lesions of urticaria pigmentosa were stroked they showed some degranulation of the mast cells with a slight influx of eosinophils [16] and in man marked local eosinophilia followed injection of histamine in the skin of atopic individuals, although this did not occur in those who were not atopic [17 18] Marked local eosinophilia followed injection of histamine in horses [2 19] but this effect was not seen in mice [20 21] guinea pigs [22] or dogs [23]

Mast cell granules contain most of the histamine in the body [24] and these granules may be released in the immediate hypersensitivity reaction [25] or in acute inflammation [26] thus producing a local eosinophilia. SHELDON and BAUER [26] showed that damage to the skin of rats produced by experimental mucormycosis caused degranulation of local mast cells within half an hour followed by a local eosinophilia in about 6 h pretreatment with compound 48/80 (a histamine liberator) caused degranulation of the mast cells, and inflammation was then not associated with a local eosinophilia. Since local eosinophilia is an uncommon accompaniment to acute inflammation, perhaps the mechanism in this case involved a hypersensitivity reaction.

There is other evidence which suggests that the function of the eosinophil is closely related to the external environment they are most numerous in the skin, stomach, and small and large intestine, and their number in these tissues is very small at birth but increases markedly during the first 6 months of life [9] However if the main function of eosinophils is inactivation of histamine, it would be expected that they would be more numerous where mast cells were plentiful this situation is found in rat skin, but in the rat tongue mast cells are numerous whereas eosinophils are few and in the rat gastrointestinal tract eosinophils are numerous but mast cells are few In view of this anomaly and since the rat stomach and small intestine are rich in histamine but poor in mast cells [24] the present authors considered it possible that the many eosinophils in the rat gastrointestinal tract were there because of a high local concentration of histamine derived from the food in support of this concept, RYTÖMAA [9] stated that a meat diet increased the number of eosinophils in the alimentary tract (though no reference to the observations was given) This point was

also made by ARCHER [27] who cited the work of BIOGAKT [28] GODLOWSKI [29 30] and TEIR *et al.* [31] however of these authors only GODLOWSKI [30] used protein-rich feeding, and although he considered that two dogs on a raw meat diet had more eosinophils in the blood and intestinal tract than did the control animals, comparison of his tables I and II indicates that the values found in the test animals were within the range found in the controls. In the present work alterations in dietary histamine and probably in endogenous histamine had no effect on the number of eosinophils present in the gastrointestinal tract.

ARCHER [2] found that an antihistamine inhibited the eosinophil response to intradermal injection of histamine in horses, thus an effect on eosinophil kinetics might have been expected when an antihistamine was continuously given to rats. The absence of such an effect, the species differences in the eosinophil response to local injection of histamine, and the finding that injection of histamine into the skin in man is followed by a local eosinophilia only in atopic individuals, suggest that the main function of eosinophils is not the inactivation of histamine.

Although ROBERTS [32] has presented evidence that eosinophils rapidly take up antigen and may thus be concerned in the formation of antibody other workers have been unable to demonstrate this effect [20 33] There is much evidence that antigen antibody complexes are eosinotactic and are phagocytosed by eosinophils [20, 25 33 34 35 36] Thus, the main function of eosinophils is probably related to the formation or disposal of antigen-antibody complexes.

Acknowledgement

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Summary

The hypothesis that the main function of eosinophils is the inactivation of histamine was investigated by using continuous infusion of tritiated thymidine to estimate the turnover and number of eosinophils in rats given normal diet, histamine-high diet, histamine-low diet, or a normal diet with antihistamine. The turnover and number of eosinophils in the bone marrow blood and tissues was not altered by any of the treatments given. These results and the known species differences in the eosinophil response to local injection of histamine suggest that the main function of eosinophils is not the inactivation of histamine.

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Cytoplasmic Shedding as a Mode of Formation of Lymphocyte-Like Blast Cells by Newt Histiocytes

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It is well known that in haemopoietic tissues of man and animals, including amphibians, particular cells are present which, due to their small dimensions and dark staining of chromatin, are variously called 'marrow lymphocytes' 'lymphocyte-like cells' 'lymphoid blast cells' [2, 7, 8, 16, 25, 29]. Introzzi and Desyllas [9], Ferrata [5], Di Guglielmo [3], Fiesch [6] and Rohr [20] had included among histiocytes a type of small lymphocyte-like cells which may be easily confused with lymphocytes for having a round compact nucleus, with a badly distinguishable nucleolus and a thin rim of slightly basophilic cytoplasm and, unlike lymphocytes, the ability to perform phagocytosis of vital dyes.

Fliedner *et al.* [7] described lymphoid blast cells appearing during haemopoietic recovery in irradiated dogs which were transfused with nitrogen-mustard treated marrow. Such cells have a large nucleus unlike that of typical lymphocytes, with a fine nuclear structure, a thin cytoplasmic rim and the capacity to synthetize DNA, thus being presumed to be endowed with stem-cell pluripotentiality. Similar cells are easily found in circulating blood of the newt *Molge Vulgaris L.* [10, 11] and are particularly numerous in early phases of the hemopoietic regeneration which ensues to refeeding the animals after a long period of starvation. Independently from any question concerning the exact role of these lymphocyte-like cells (stem cell pluripotentiality?) we have had the opportunity of observing and describing in the present paper an obsolete though not infrequent mode of formation of lymphoid cells by histiocytes which were shedding in the surrounding medium large fragments of their cytoplasm.

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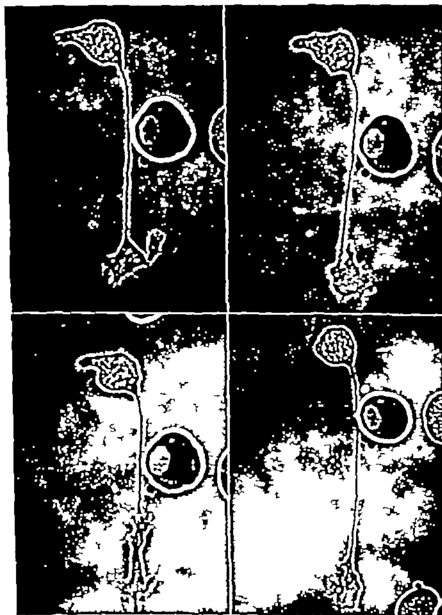


Fig 2. Progressive shedding of large cytoplasmic fragment by newt histiocyte. Frames from phase-contrast cine-recording.]

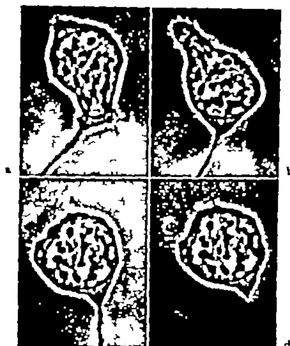


Fig 3. Same cell as in figure 2. a–c The progressive stretching and thinning of the connecting cytoplasmic bridge causes the loss of a large portion of cytoplasm from the histiocyte. d Retraction of the remnant of the cytoplasmic bridge.

of migratory movement, but the customary shifting in the same direction of the remaining body of the cell does not follow The cell itself remains fixed in its position.

Still the pseudopodium is provided with a very marked autonomous motility appearing as it was actively pulling on its connection with the cell (fig 2) As a consequence, a progressive thinning of the reunion trait is being operated, until the cytoplasmic bridge is broken and the pseudopodal mass is completely detached

While the cell retracts back the remnant of the cytoplasmic bridge and acquires a round shape, with the remaining cytoplasm forming a thin rim around the nucleus (fig 3) the fragment of cytoplasm lost by the cell in the medium performs a rather intense but apparently uncoordinated locomotory activity (fig 4) The final step of the whole process has been the production of a small cell, with a high nucleo-cytoplasmic ratio still provided with histiocytic potentialities, but remarkably resembling a lymphocyte.

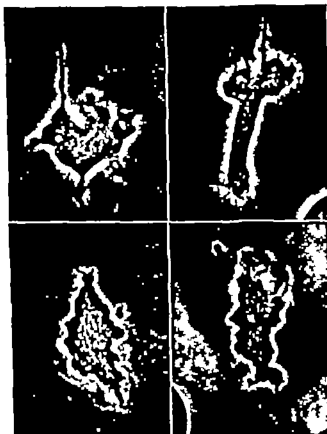


Fig 4 Fate of the portion of cytoplasm lost by the histiocyte. The detached fragment performs uncoordinated locomotory movements in the medium before dissolution.

Discussion

Cytoplasmic shedding which was first described by RANVIER [19] has already been the subject of several reviews [26 12 28, 13 17 18] the phenomenon being variously related to water and food transport or plasma formation or globulin releasing by mononuclear cells [24 26] Until now the interest of investigators was primarily focused on the probable fate of released cytoplasmic droplets and only in part on the becoming of the original cell. According to DOWNEY and WEIDENREICH [4] and to SHIELDS [26] many of the cells undergoing cytoplasmic shedding may eventually differentiate into specific cell types, namely lymphocytes, plasmocytes, etc.

Our phase-contrast cinerecords have now presented the visual evidence in living cells that such a phenomenon may actually occur at least as far as the capacity of histiocytes is concerned of assuming a lymphocyte like appearance and is not an artifact of fixation and staining procedures The resulting cells, though preserving their histiocytic pattern of nuclear chromatin may be easily confused with lymphocytes according to their dimension and the nucleo-cytoplasmic ratio The signification of such a phenomenon is not clear but we find it very interesting that these histiocyte-derived lymphocyte-like cells were very numerous in peripheral blood of newts during early phases of post-starvation haemopoietic regeneration and immediately preceding the appearing in the blood of large undifferentiated blast cells which probably derive from them and which subsequently differentiate into actively dividing erythroblasts.

Lymphocyte like cells are in this rather similar to lymphoid blast cells which were regularly observed in haemopoietic organs by Magliulo [14] and Magliulo *et al* [15] during early phases of hemopoietic regeneration in irradiated monkeys which had been transfused with autologous and homologous marrow More recently similar reports were presented also by Thomas [27] and by Fliedner [7] strongly suggesting though not proving a genetic relationship between differentiated hemopoietic cells and those primitive lymphoid blast forms which we have demonstrated may in certain circumstances originate from histiocytes by means of cytoplasmic shedding It is interesting enough that lymphocyte-like cells may also originate in peripheral blood cultures by means of the mechanism of cytoplasmic shedding from large pyroninophilic blasts [1] themselves originating from phytohemoagglutinin stimulated small lymphocytes.

Summary

In newts undergoing hemopoietic regeneration after long period of starvation, circulating histiocytes were shown t shed large fragments of their cytoplasm, eventually differentiating into lymphocyte-like cells. The present investigation which was conducted by means of phase-contrast cinemicrography on living cells, presents the visual evidence of an obsolete morphogenetic pattern of cells which may be provided of stem-cell pluripotentiality

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Panmyelose mit Panhämozytopenie

H. Löffler und L. D. Leder

Unter dem Begriff «myeloproliferierende Mischformen» hat Di Guglielmo [5] solche Myelosen aus dem Komplex der myeloproliferativen Krankheiten [3] herausgehoben, bei denen mehrere Zellreihen des Knochenmarkes vom pathologischen Prozess ergriffen sind. Die Beteiligung mehrerer Zellreihen an derartigen Hämoblastosen kann entweder konstant – also während des gesamten Krankheitsverlaufes – oder nur phasenhaft nachweisbar sein. Im einzelnen unterscheidet Di Guglielmo erythroleukämische, erythromegakaryozytäre, leukomegakaryozytäre und erythroleukomegakaryozytäre Myelosen.

Beim letztgenannten Krankheitsbild, welches er auch als Panmyelose bezeichnet, beschreibt Di Guglielmo [4, 5] zwei Formen: 1. eine über Jahre verlaufende chronische, totale, hyperplastische Panmyelose, die äusserst selten sei und die sich durch eine konstante Panzythämie mässigen Grades (ähnlich der Polycythaemia vera rubra) und zusätzlich durch die Ausschwemmung von unreifen Erythroblasten, unreifen Granulozyten sowie kleinen Megakaryozytenfragmenten auszeichnet; 2. erwähnt er eine «fast stets akute, unvollständige, hyperplastische Panmyelose». Eine nähere Charakterisierung des letztgenannten Typus gibt Di Guglielmo allerdings nicht.

Die grosse Seltenheit derartiger Hämoblastosen veranlasst uns, im folgenden über einen Patienten zu berichten, dessen Krankheitsbild nach den Ergebnissen zytologischer, fermentzytochemischer und autoptischer Untersuchungen einer Panmyelose entspricht.

Kasuistik

Klinische Befunde

66jähriger Mann. 1918 Diphtherie und Ruhr. 1959 Operation eines Ösophagusdivertikels. Sonst keine ernsthaften Erkrankungen. Familienanamnese o.B.

Seit Januar 1962 bei körperlicher Belastung Atemnot und Schwindelgefühl. Ende Juli 1964 schwarzer Stuhl. Deutliche Verschlechterung des Allgemeinbefindens. Zunahme von Atemnot und Schwindelgefühl, mehrmals Ohnmachten.

Erste stationäre Aufnahme am 11.8.1964. Auffällige Blässe, reduzierter Allgemeinzustand. Leber, Milz und Lymphknoten nicht vergrößert. Keine hämorrhagische Diathese. Im Stuhl bei mehrfacher Kontrolle kein Blut nachweisbar. Laborbefunde: BSG 25/60 mm. Serumtransaminasen, alkalische Phosphatase, Bilirubin, Serumlabilitätsproben, Gesamtprotein und Elektropherogramm unauffällig. Röntgenologisch zwei Divertikel im unteren Sigma; Magen-Darm-Trakt sonst o.B. Weiterhin schwere normochrome Anämie, Leukozytengesamtzahl an der unteren Normgrenze. Therapie: Insgesamt 9 Bluttransfusionen zu je 500 ml. Anstieg des Hämoglobins von 3,3 auf 11,4 g%. BSG 11/28 mm. Am 11.9.1964 Entlassung.

Erneute Hospitalisierung bei im wesentlichen unverändertem klinischem Befund wegen Zunahme der subjektiven Beschwerden am 21.10.1964. Röntgenologisch an Ösophagus, Magen, Colon, Nieren, Gallenblase und Thoraxorganen kein pathologischer Befund. Hämoglobin 7,9 g%. Leukozytengesamtzahl zwischen 3100 und 6000/mm³ schwankend. Thrombozyten 59000/mm³. Serum-Eisen 218–319 µg%. HbE 30,6. Retikulozyten 2–10 ‰. Osmotische Resistenz der Erythrozyten: 0,50%–0,34% NaCl. Gerinnungsuntersuchungen

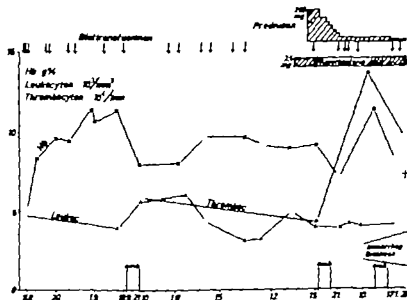


Abb. 1. Verhalten von Hämoglobin, Leukozyten und Thrombozyten während des Krankheitsverlaufes.

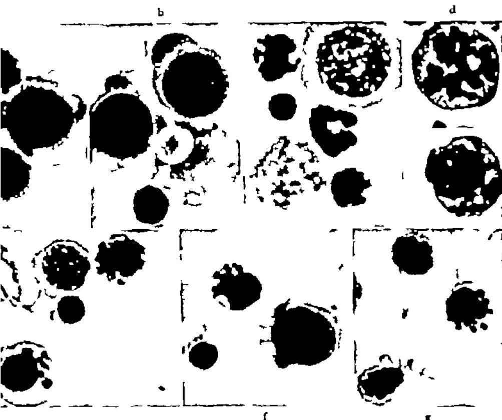


Abb. 2. ( ) und (b) Junge, megaloblastäre Erythroblasten mit pseudopodienartigen Plasmaausläufern. ( ) Pathologische Erythroblastenmitosen mit erklumpten Chromosomen. Links unten ein Promyelozyt. (d) bis (g) Pathologische Erythroblastenmitosen sowie Erythroblasten mit paranukleären Chromatinbrocken. Sternalpunktat. Pappenheim-Färbung (×1400).

Quick 108%, Gerinnungszeit 2'30" Blutungszeit 1'5 Faktor II 70%, Faktor V 114%, Faktor VII 86%. Über das Verhalten von Hämoglobin, Leukozyten und Thrombozyten während des Krankheitsverlaufes unterrichtet die Abb. 1 Die Sternalpunktion (28.10.1964 und 7.12.1964, siehe unten) ergab die Diagnose Panmyelose. Therapie Ab 8.12. täglich 2,5 mg Amethopterin sowie Prednison mit einer anfänglichen Dosis von 200 mg/die, die kontinuierlich bis auf 25 mg/die reduziert wurde. In der Zeit vom 30.10. 14.12.1964 insgesamt 7 Bluttransfusionen zu je 500 ml. Bei der Entlassung am 15.12. Hämoglobin 9,1 g%, Leukozyten 4000/mm³ Thrombozyten 42000/mm³

Dritte stationäre Aufnahme am 2.1.1965. Hb 7,0 g% bei 2,53 Mill. Erythrozyten Leukozyten 2900/mm³ Serumeisen 182 und 217 μg%. Weiterbehandlung mit täglich 25 mg Prednison sowie 2,5 mg Amethopterin. Außerdem in der Zeit vom 2.1. 14.1.1965 insgesamt 4 Bluttransfusionen zu 500 ml. Entlassung am 14.1.1965.

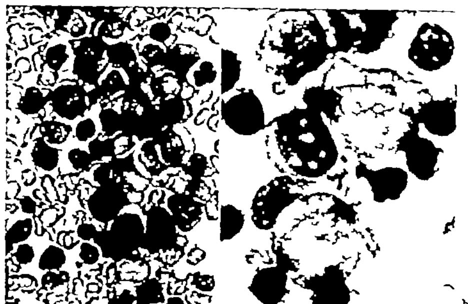


Abb. 3 ( ) Blasten, Promyelozyten und einzelne sogenannte Pseudopelgerformen. (b) Blasten, Promyelozyten, dazwischen Erythroblasten mit stark verklumpten und verdichteten pyknoseartig veränderten Kernen. Sternalmarkausstrich (× 1400)

Vierter stationärer Aufenthalt in der Zeit vom 17.1.–21.1.1965 wegen Fieberschüben und ausgedehnten flächenhaften Hautblutungen. Schwere, durch antibiotische Behandlung und Herzkreislauftherapie nicht mehr zu beherrschende Bronchopneumonie. Am 21.1.1965 Exitus letalis.

Morphologische Befunde

Im *peripheren Blutausstrich* zeigten die Erythrozyten eine hochgradige Anisozytose und Poikilozytose sowie eine ausgeprägte Hypochromasie. Häufig waren sie basophil punktiert und enthielten Cabotsche Ringe. Auf 100 Leukozyten fanden sich maximal 2 Normoblasten, deren Zytoplasma vakuolisiert, zum Teil optisch leer erschien. Im weißen Differentialblutbild wurden maximal 3% Myeloblasten gezählt. Der Anteil der Lymphozyten lag im Normbereich. Die neutrophilen Granulozyten waren vermindert und zum großen Teil pathologisch verändert. Es fanden sich sogenannte Pseudopelgerformen, weiterhin vereinzelt übersegmentierte und polyploide Neutrophile mit eigenartig verklumpten Kernen. Gelegentlich waren im Zytoplasma der stab- und segmentkernigen Neutrophilen Vakuolen und Doehle-Körperchen vorhanden. Auch die Thrombozyten waren atypisch. Sie zeigten eine erhebliche Polymorphie, und es fanden sich Riesenplättchen sowie ungranulierte Formen.

Bei den beiden *Sternalmarkuntersuchungen*, die wir am 28.10. und am 7.12.1964 durchführten, wurden so weitgehend identische Veränderungen gefunden, dass wir sie zusammenfassend darstellen. Die Ausstriche waren recht zellreich. An der Zellvermehrung waren alle drei blutbildenden Zellreihen beteiligt. Am stärksten trat die Erythropoiese hervor. Es fanden sich zahlreiche Anomalien, von denen alle Reifungsstufen betroffen waren (Abb. 2): megaloblastäre Formen, zwei- und mehrkernige Erythroblasten, atypische

Mitosen sowie sehr häufig Kernverklumpungen und Chromatinabsprengungen. Bei manchen Erythroblasten war das Zytoplasma von kleinen runden Chromatinbrocken angefüllt. Solche Veränderungen waren manchmal kaum von pathologischen Mitosen zu unterscheiden. Manche reiferen Erythroblasten zeigten ein auffällig schmales Zytoplasma, andere eine Vakuolisierung. Auch bei den reiferen Erythroblasten waren die Kerne verändert, und zwar besassen sie häufig ein dichtes, klumpiges, fast strukturloses Chromatin, so dass der Eindruck einer Pyknose entstand (Abb. 5b).

In der granulozytären Reihe fielen insbesondere Blasten und Promyelozyten auf (Abb. 5b), die herdförmig zusammenlagen und erhebliche Kernpolymorphien und Granulationsanomalien aufwiesen. Die reiferen Granulozytenvorstufen entsprachen zum Teil sogenannten Pseudopelgerzellen (Abb. 3a), vereinzelt sah man Riesenformen, wie sie bei megaloblastärer Anämie auftreten.

Die reichlich vorkommenden Megakaryozyten (Abb. 4) liessen erhebliche Grössendifferenzen erkennen. Zytologisch fanden sich zahlreiche Atypien und Abweichungen von der Norm. Neben Megakaryozyten mit stark segmentierten oder gelappten Kernen sahen wir Knochenmarkriesenzellen mit sehr stark verklumptem Chromatin, ähnlich wie bei einer Kernpyknose. Das Zytoplasma der Megakaryozyten war in den meisten Fällen basophil und nicht granuliert sowie von verschieden grossen Vakuolen durchsetzt. Die Zellgrenzen waren oft nicht klar erkennbar, sondern stark zerklüftet, so dass der Eindruck eines scholligen Plasmazerfalls entstand. Innerhalb von basophilen Zytoplasmabezirken sah man schliesslich ungefärbte oder zart rötlich gefärbte Areale. Auch bei den Megakaryozyten fielen uns zahlreiche Mitosen mit Chromosomenabsprengungen und Chromatinverklumpungen auf.

Cytochemische Befunde

Eisennachweis. 60% der Erythroblasten der Sternalausstriche enthielten freies Eisen in Form von kleinen Granula. Dabei waren die Granula gegenüber der Norm vergröbert, sie lagen meist perinukleär. Weiterhin war eine erhebliche Vermehrung von eisenspeichernden Retikulumzellen zu konstatieren.

Perjodsäure-Schiff-Reaktion (PAS). 1–12% der Erythroblasten des Knochenmarkes fand sich eine schwache diffuse positive Reaktion. Die Megakaryozyten waren dagegen häufig negativ, zum Teil auch schwach homogen positiv. Von den Zellen der Granulozytopoiese reagierten die Blasten negativ. In den Promyelozyten und den weiteren Ausreifungsstadien fiel die Reaktion zwar positiv aus, aber schwächer als normal.

Alkalische Phosphatase. In den Sternalausstrichen zeigten Kapillarendothelien sowie einzelne reife Granulozyten eine positive Reaktion. Im peripheren Blutbild (Untersuchung vom 7. 12. 1964) waren 38% der reifen Granulozyten phosphatase-positiv, wobei ein Index von 44 ermittelt wurde.

α-Naphthylacetat-Esterase. Am stärksten positiv reagierten die Retikulumzellen. Bei den Erythroblasten aller Reifungsstadien war die Aktivität ganz erheblich gesteigert, wobei das Reaktionsprodukt diffus im Zytoplasma verteilt erschien. Auch die Erythroblastenmitosen wiesen diese Aktivierungssteigerung auf. In den Megakaryozyten war gelegentlich eine ausserordentlich grobgranuläre Ablagerung von Reduktionsprodukt vorhanden. Die Granulozytopoiese zeigte keine Besonderheiten.

Autoptische Befunde (Sekt.-N. 73/65)

Makroskopische Befunde. Panmyelose: Dunkelrotes Mark im gesamten Femur, in den Wirbelkörpern und im Sternum. Geringgradige Vergrösserung von Leber und Milz. Hämorrhagische Diathese: Bis zehnpfennigstückgrosse Blutungen in der Magenschleimhaut; flächenhafte Blutungen in der Nierenbeckenschleimhaut beiderseits und im Nierenparenchym rechts; pfennigstückgrosse, subintimale Blutung im Bereich der Aorta abdominalis; flächenhafte Hautblutungen im Bereich des Stammes und der Extremitäten; kleine, nicht ganz frische

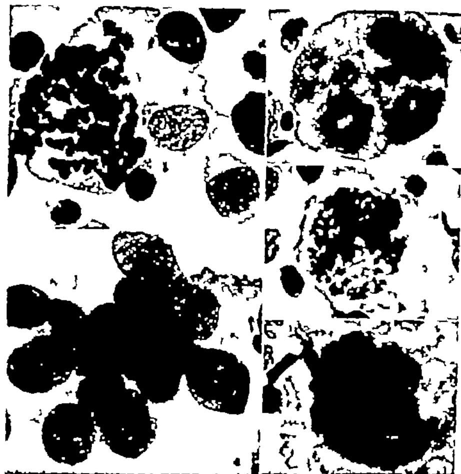


Abb. 4 Atypische Megakaryocyten und Megakaryocytenmitosen. Sternalausstrich. Pappenheim-Färbung (×1000)

Hirnblutungen im Putamen-Klaustrumbereich links. Schwere chronische katarrhalische Tracheobronchitis. Geringgradige diffuse Bronchiektasen beiderseits. Schwere fibrinreiche Pneumonie beiderseits. Fibrinöse Pleuritis beiderseits, rechts stärker als links. Mäßig *schweres chronisches substantielles Lungenemphysem. Dilatation der rechten Herzkammer* Leberverfettung. Lipoidentspeicherung der Nebennierenrinde beiderseits. Geringgradige Arteriosklerose der Aorta. Schwere Coronararteriensklerose mit weitgehendem Verschluß des R. interventricularis der A. coronaria sinistra. Dilatation der linken Herzkammer. Schweres Lungenödem. Cholesteatose der Gallenblase. Adenomatöse Prostatahyperplasie. Geringgradige Balkenblase

Histologische Befunde. Im *Knochenmark* von Wirbel, Sternum und Femur verhielt sich die Spongiosa regelhaft, die Silberfasern waren nicht vermehrt. Die Fettzellen waren fast vollständig geschwunden. Bei schwacher Vergrößerung war bereits eine merkwürdige und ungewöhnliche Vielfalt von Zellen auffällig: Neben sehr kleinen kamen sehr große, neben

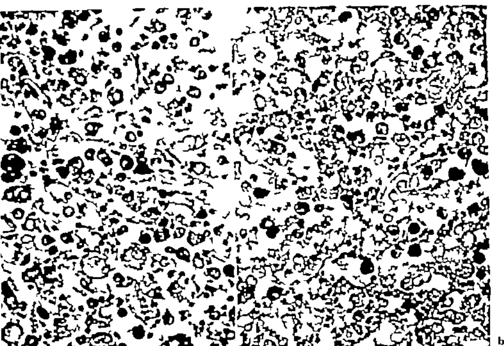


Abb. 5. Schnittpräparat vom Femurmark. ( ) Schwund der Fettzellen. Starke Vermehrung von atypischen Megakaryozyten und zum Teil mehrkernigen Erythroblasten. Giemsa. (b) Nachweis der Naphthol-AS-D-Chloracetat-Esterase am Paraffinschnitt. Die atypischen, promyelozytenähnlichen Zellen der neutrophilen Granulopoiese heben sich durch ihre positive Reaktion (schwarz, im Original leuchtend rot) heraus (×350)

stark basophilen oxyphile Elemente mit teils runden, teils auch bizarren, gelappten Kernen vor. Viele der Zellen waren mehrkernig.

Bei stärkerer Vergrößerung (Abb. 5a) waren Megakaryozyten, Erythroblasten und Granulopoesezellen zu unterscheiden. Die Megakaryozyten lagen in lockeren Gruppen und besaßen häufig sehr weites, teils basophiles, teils oxyphiles, leicht gekörntes Plasma. Oft waren ihre Plasmaränder ausgesprochen unscharf begrenzt. Die Megakaryozytenkerne zeigten ein lockeres, helles Chromatingerüst und waren oft bizarr geformt mit Lappungen und Segmentierungen aller nur erdenklichen Formen. Nukleolen waren deutlich erkennbar, die Kernmembranen meist scharf ausgeprägt.

Die Erythropoesezellen standen zahlenmäßig durchaus im Vordergrund. Es waren alle Reifungsstufen bis zum oxyphilen Normoblasten in bunter Mannigfaltigkeit zu erkennen, doch bestanden erhebliche Größenunterschiede. Während die Normoblasten durch ihr oxyphiles Plasma relativ gut erkennbar waren, gelang die sichere Identifizierung von unreifen basophilen Erythropoesevorstufen selbst im Giemsa-Präparat nicht in allen Fällen. Unter den unreifen Erythropoesezellen fielen häufig zweikernige Exemplare auf. Viele Erythropoesezellen waren in Mitose begriffen. Dabei fanden sich im Plasma nicht selten abgesprengte Kernteile. Besonders auffällig war das Fehlen der normalerweise immer vorhandenen und für die Erythropoese typischen betont gruppenförmigen Lagerung der Zellen.

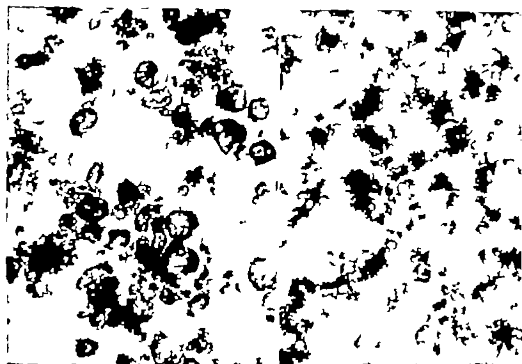


Abb. 6. Schnittpräparate vom Femurmark. (a) Nachweis der α-Naphthylacetat-Esterase. Atypische Erythroblasten stark positiv mit typischer perinukleärer Reaktion (schwarz, im Original rotbraun). Dazwischen einige sehr stark positive Plasmaausläufer von Retikulumzellen (×560). (b) Nachweis der sauren Phosphatase. Im wesentlichen sind die Retikulumzellen positiv (schwarz, im Original rot), daneben wenige Proerythroblasten mit punktförmiger paranukleärer Reaktion (z. B. rechts oben) (×350).

Die Granulopoiesezellen traten im Präparat zahlenmäßig zurück. Segmentkernige, stabkernige Metamyelozyten und Myelozyten waren kaum vorhanden. Dagegen konnten einzeln liegende typisch gestaltete Promyelozyten identifiziert werden. Auch Vorstufen der Eosinophilen kamen gelegentlich vor.

Weiterhin waren Retikulumzellen in mäßiger Anzahl, einige Plasmazellen sowie Lymphozyten eingestreut. Gewebsmastzellen kamen nicht vor.

In der *Milz* fanden sich innerhalb der Sinus nur wenige Infiltrate aller drei blutbildenden Zellsysteme. Am auffallendsten stachen die Megakaryozyten hervor, die die gleichen pathologischen Veränderungen zeigten wie im Knochenmark. Im Gegensatz zu den Verhältnissen im Knochenmark waren unter den Myelopoiesezellen relativ reichlich reife Granulozyten vorhanden. Erythropoiesezellen kamen nur vereinzelt vor. Die Pulparetikulumzellen wiesen eine starke Hämosiderose auf. Plasmazellen waren reichlich vorhanden.

In der *Lunge* bestand eine erhebliche fibrinreiche Bronchopneumonie mit Infiltraten durch gelapptkernige neutrophile Granulozyten, von denen ein grosserer Teil als Pseudopelgerformen vorlag. In den Alveolen waren ausserdem grosse Mengen einer eiweissreichen Flüssigkeit im Sinne eines Lungenödems vorhanden. Vielfach traf man auf kleine Blutungsherde.

In *Leber, Lymphknoten* vom Lungenhilus, *Tonsillen, Niere* und *Herz* fielen keine Besonderheiten auf, insbesondere keine leukämischen Infiltrate.

Histochemische Befunde Im *Knochenmark* reagierten die Megakaryozyten bei der PAS-Färbung sämtlich schwach diffus-positiv. Die Reaktion war jedoch erheblich schwächer als normalerweise. Die Megakaryozyten zeigten weiterhin eine schwache Reaktion beim Nachweis von α-Naphthylacetat-Esterase von Naphthol-AS-acetat Esterase sowie von saurer Phosphatase. Naphthol-AS-D-Chloracetat-Esterase und alkalische Phosphatase besaßen die Megakaryozyten nicht.

Die Erythropoiesezellen reagierten beim α-Naphthylacetat Esterase Nachweis außerordentlich stark positiv (Abb. 6a) und erreichten fast den Aktivitätsgrad der Reticulumzellen, die die am stärksten positiven Knochenmarkzellen darstellen. Besonders intensiv färbten sich große, den Proerythroblasten an die Seite zu stellende Elemente an. Sie zeigten bereits nach einer Inkubationszeit von nur 2 min eine deutliche Reaktion. Im Unterschied zum Sternalpunktat war die Fermentaktivität vor allem auf eine schmale, den Kern unmittelbar wie eine Schale umgebende Plasmazone konzentriert, wie dies für normale Erythropoiesezellen hochcharakteristisch ist. Beim Nachweis der sauren Phosphatase reagierte ein kleiner Teil der besonders unreifen Erythroblasten mit einer deutlichen Anfärbung innerhalb eines paranukleären Plasmabezirkes (Abb. 6b). Naphthol-AS-acetat Esterase, Naphthol-AS-D-Chloracetat-Esterase und alkalische Phosphatase konnte in den Erythropoiesezellen nicht nachgewiesen werden.

Mit dem Nachweis der Naphthol-AS-D-Chloracetat Esterase stellten sich die neutrophilen Granulopoiesezellen elektiv dar. Sie kamen (Abb. 5b) nur in relativ geringer Anzahl vor und entsprachen etwa den Promyelozyten. Abweichend von den normalen Promyelozyten besaßen sie jedoch unregelmäßig gestaltete Kerne. Manchmal waren sie auch zweikernig. Beim Nachweis der α-Naphthylacetat Esterase, der Naphthol-AS-acetat-Esterase, der sauren und der alkalischen Phosphatase verhielt sich die Granulopoiese unauffällig.

An den übrigen Organen konnten durch die zytochemischen Untersuchungen keine Befunde erhoben werden, die über diejenigen Gesichtspunkte hinausführten, die bereits durch die histologische Untersuchung gewonnen worden waren.

Diskussion

Die klinischen Befunde unseres Patienten erlaubten zunächst ohne die Untersuchung des Sternalpunktates keine endgültige Diagnose. Zwar bestand von Beginn der Erkrankung an eine Panhämozytopenie mit Anämie, Thrombozytopenie und Leukozytenwerten, die an der unteren Normgrenze lagen oder gering erniedrigt waren, und im Differentialblutbild wurden einzelne Blasten und Erythropoiesezellen gefunden. Doch wiesen diese Befunde, wie auch die qualitativen Veränderungen der neutrophilen Granulozyten mit Pseudopelgerformen, Übersegmentation und Polyploidie und wie die morphologischen Veränderungen der Erythrozyten und Thrombozyten lediglich auf eine schwere Schädigung der Hämatopoese hin, ohne eine sichere Diagnose zu erlauben.

Erst bei der Untersuchung des Sternalmarkes konnte die Erkrankung in ihrem Wesen klar erkannt werden. Es fand sich eine schwere Hyperplasie aller drei blutbildenden Zellreihen mit hochgradigen

Atypien in der Erythropoiese, mit atypischen Blasten und Promyelozyten in der Granulopoiese *und vor allem mit reichlich polymorphen*, unreifen und atypischen Megakaryozyten. Die Erythroblasten und die Megakaryozyten fielen besonders auf Einzelne Erythroblastenkerne waren fragmentiert, neben noch erhaltenen Kernen fanden sich An *sammlungen von «Chromatinkugeln» und ausserdem sah man sowohl* in den Erythroblasten als auch in den Megakaryozyten zahlreiche verschiedengestaltige Mitoseanomalien Zytochemisch imponierte eine deutliche Vermehrung der Sideroblasten mit vergröberten Eisen granula. Ferner war die α Naphthylazetat Esterase Aktivität der Erythroblasten erheblich gesteigert. Bei der PAS-Reaktion ergab sich allerdings keine wesentliche Erhöhung des Anteiles positiver Erythroblasten gegenüber der Norm. An Hand dieser Befunde konnte die Diagnose einer Panmyelose sicher gestellt werden.

Bei der Autopsie wurde eine eindeutige Hyperplasie des Knochenmarksparenchyms mit einem fast vollständigen Schwund der Fett zellen festgestellt. Ausserdem fanden sich in der Milz Infiltrate von Megakaryozyten, von Granulopoiesezellen und von Erythroblasten Im Knochenmarkschnitt fiel vor allem die ungewöhnliche Vielfalt der Zellen und die sehr starke Vermehrung von atypischen Mega karyozyten auf, die an manchen Stellen sehr dicht gelagert waren. Zahlenmäsig beherrschte jedoch – wie im Knochenmarkausstrich auch – die hochgradig polymorphe und atypische Erythropoiese mit vielen mehrkernigen Zellen und reichlich Mitosen das Bild. Die Granulopoiese stand dagegen quantitativ zurück, war aber auch im histologischen Schnitt erkennbar pathologisch verändert und insbesondere durch die Anwendung der Naphthol AS-D-Chlorazetat Esterase Reaktion am Paraffinschnitt [10] gut darzustellen. Damit war auch in Anbetracht der autoptischen Befunde an dem Vorliegen einer Hämoblastose nicht zu zweifeln, wie aus der schweren Infiltration des Knochenmarkes mit Verdrängung der Fettzellen und aus dem Nachweis von atypischen Megakaryozyten, Granulopoiesezellen und Erythropoiesezellen in der Milz hervorgeht. Dass es sich im vorliegenden Falle zweifellos um eine Panmyelose handelt, zeigen die schweren morphologischen Veränderungen an allen drei Knochenmarkszellreihen.

Ausser morphologischen Befunden, die das Vorliegen eines malignen Geschehens bewiesen, waren auch entsprechende zytochemische Befunde vorhanden, wie sie bei anderen malignen Hämoblastosen vorkommen So imponierte eine deutliche Vermehrung von Sidero-

blasten mit vergröberten Eisengranula sowie eine Vermehrung von eisenspeichernden Retikulumzellen. Beide Befunde sind als Ausdruck einer Eisenverwertungsstörung zu betrachten und sie kommen – abgesehen von der sideroachrestischen Anämie – auch bei Erythrämien und Erythroleukämien vor [7 15 8] Im übrigen ist die bei unserer Beobachtung vorliegende Eisenverwertungsstörung im Rahmen einer Abhandlung über die pathologische Anatomie der sideroachrestischen Anämie ausführlicher besprochen [12] so dass sich ein weiteres Eingehen auf diese Problematik erübrigt.

Die erheblich gesteigerte α Naphthylazetat Esterase Aktivität der Erythroblasten stützt ebenfalls die Annahme einer malignen Entartung der roten Reihe, da ähnliche Aktivitätserhöhungen bisher nur bei Erythrämie und Erythroleukämie beobachtet wurden wenn man von der perniziösen Anämie absieht [16 13 14 11] Offenbar ist dieser zytochemische Befund immer dann zu erheben, wenn eine in hohem Masse ineffektive Erythropoiese vorliegt.

Die PAS-Reaktion erbrachte allerdings keinen Hinweis auf das Vorliegen einer malignen Veränderung innerhalb der Erythropoiese denn der Anteil positiver Erythroblasten war gegenüber der Norm nicht wesentlich erhöht. Im allgemeinen wird eine deutlich gesteigerte PAS-positive Reaktion eines grossen Teiles der Erythroblasten als sehr charakteristischer Befund bei reiner Erythrämie und bei Erythroleukämie angesehen [1 18, 15] Dies konnten wir bei eigenen Untersuchungen an 6 Patienten mit Erythroleukämie und an einem Patienten mit erythrämischem Terminalstadium bei chronischer myeloischer Leukämie bestätigen Bei subakut oder chronisch verlaufenden Neoplasien der Erythropoiese kann die gesteigerte PAS-Reaktion der Erythroblasten aber auch fehlen oder nur sehr gering ausgeprägt sein [22, 7] Daraus ergibt sich, dass nur der positive Befund als Stütze für die Annahme einer malignen Erkrankung der roten Reihe anzusehen ist, während umgekehrt ein negativer Befund wie er in unserem Falle erhoben wurde, eine solche Möglichkeit keineswegs ausschliesst.

Bei dem von uns beschriebenen Krankheitsbilde handelt es sich um eine besondere Hämoblastose die von anderen myeloproliferativen Erkrankungen, wie der Polycythaemia vera rubra der Osteomyelosklerose und Myelofibrose einschliesslich der Übergangsformen zu chronischen myeloischen Leukämien und von der sogenannten Panmyelopathie mit hyperplastischem Mark abgegrenzt werden kann Von diesen Krankheitszuständen unterscheidet sich unsere Panmyelose durch die sehr schweren Zellatypien. Von der Osteomyelo-

sklerose und von der Myelofibrose ist die Panmyelose durch die fehlende Spongiosa und Faservermehrung abgegrenzt. Gegen das Vorliegen einer Polycythaemia vera rubra sprechen die klinischen Befunde. Eine Panmyelopathie mit hyperplastischem Mark, wie sie in extrem seltenen Fällen als Initialstadium einer chronischen Benzolvergiftung beschrieben ist [2] kann anamnestisch ausgeschlossen werden. Andere Fälle von Panmyelopathie, die meist ätiologisch nicht zu klären sind und vielfach als Autoimmunreaktion gedeutet werden kommen ebenfalls nicht in Frage, da bei einer solchen Annahme die schweren zytologischen und zytochemischen Veränderungen unverständlich blieben, die eindeutig auf eine maligne Proliferation der Knochenmarkszellen hinweisen. Im übrigen kann keinesfalls ausgeschlossen werden, dass sich hinter manchen Fällen von derartigen Panmyelopathien mit hyperplastischem Mark in Wirklichkeit Panmyelosen verbergen.

Die Befunde des von uns beobachteten Patienten weichen in verschiedenen Punkten von der Beschreibung des Panmyelosetypus I von Di Guglielmo [5] ab. So fehlt z.B. die Panzythämie, und unreife Zellen der Hämopoiese wurden nur bei längerem Suchen in geringer Zahl im peripheren Blutbild gefunden. Schliesslich erstreckte sich der Krankheitsverlauf auch nicht wie bei den Fällen Di Guglielmos über mehrere Jahre, sondern war sehr kurz. Ob eine Ähnlichkeit mit der zweiten von Di Guglielmo erwähnten Verlaufsform der Panmyelose besteht, die er eine «fast stets akute, unvollständige, hyperplastische Panmyelose» nennt, vermögen wir nicht zu entscheiden, da Di Guglielmo eine genaue Charakterisierung dieses Krankheitsbildes nicht gegeben hat.

Von manchen werden unter dem Terminus Panmyelose Krankheiten verstanden, die von unserer Beobachtung in vielen Einzelheiten abweichen und die im allgemeinen auch nicht ohne weiteres als maligne Hämoblastosen angesehen werden. So belegen Herbeuval und Mitarbeiter [9] die Osteomyelosklerose und verwandte Krankheitsbilder mit dem Ausdruck «panmyélémie» und Rappaport [19] fasst unter dem Begriff Panmyelose die Polycythaemia vera und die «Myelosklerose mit myeloischer Metaplasie» zusammen. Wir sind dagegen der Meinung, dass die Bezeichnung Panmyelose solchen Hämoblastosen vorbehalten bleiben sollte, die eindeutig Zeichen einer malignen Proliferation aller drei blutbildenden Systeme in Form von schweren Zellatypien etc. aufweisen, wie das bei unserer Beobachtung der Fall ist. Im übrigen ist das von uns beschriebene Krankheitsbild

durch den völlig abweichenden klinischen Verlauf und den abweichenden pathologisch-anatomischen Befund sowohl von der Osteomyelosklerose als auch von der Polycythaemia vera klar abgetrennt.

Unsere Beobachtung könnte die durch zytogenetische Untersuchungsergebnisse gestützte Ansicht von einer gemeinsamen Stammzelle für die Erythropoese, die Granulopoiese und die Megakaryozytopoiese weiter unterbauen [20, 21, 23], eine Ansicht, die u. a. aus dem Vorkommen des für die chronische myeloische Leukämie typischen Philadelphia-Chromosoms auch in Erythroblasten und Megakaryozyten abgeleitet ist. Eine solche gemeinsame Stammzelle wird auch von HAYHOE und Mitarbeitern [6] anhand zytologischer und zytochemischer Untersuchungsergebnisse bei unreifzelligen Leukosen diskutiert. Allerdings ist auch die Möglichkeit zu erwägen, dass Knochenmarkszellen – vor allem, da sie genetisch untereinander nahe verwandt sind – gleichartig anfällig gegenüber geschwulstauslösenden Noxen sind. Damit wäre z. B. denkbar, dass ein solches Agens bei allen Zellreihen gleichartige Veränderungen hervorruft, ohne dass eine gemeinsame Stammzelle unbedingt vorliegen muss.

Zur Frage der Therapie ist zu erwähnen, dass wir mit kleinen Dosen von Amethopterin, wie sie von MOESCHLIN [17] zur Behandlung von Erythroleukämien empfohlen werden und wie sie auch von uns mit gutem Erfolg bei einem Fall von Erythroleukämie eingesetzt werden konnten, in Kombination mit Prednison bei unseren Patienten keinen sichtbaren Erfolg hatten. Allerdings konnten wir die Behandlung nur 14 Tage lang durchführen. LÜDIN [13a] hat bereits 1950 einen deutlichen Effekt des Folsäureantagonisten Aminopterin bei Erythroleukose beschrieben.

Zusammenfassung

Es wird über einen 66jährigen Patienten berichtet, der das Krankheitsbild einer Panmyelose mit Panhämozytopenie bot. Bei der bioptischen und autoptischen Untersuchung wurde eine schwere Hyperplasie des Knochenmarkes festgestellt, an der die Erythropoese, die Granulopoiese und die Megakaryozytopoiese beteiligt waren. Alle drei Zellreihen wiesen überdies schwere zytologische Atypien auf. In der Milz fanden sich extramedulläre Blutbildungsherde. Die zytologischen und zytochemischen Befunde werden im Hinblick auf die allgemeine hämatologische Bedeutung solcher Fälle besprochen, und es werden diagnostische, differential-diagnostische und therapeutische Fragen gestreift.

Summary

This paper reports a case of malignant panmyelosis with panhemocytopenia, which occurred in a 66-years-old patient. At biopsy and autopsy the bone marrow revealed a heavy hyper

phasis of the blood forming parenchyma including erythropoesis, granulopoiesis, and megacaryopoiesis as well. There were in addition, many cytological atypias in each of the three bone marrow cell strains. The cytological and cytochemical observations are discussed with respect to the general hematological importance of such cases, and problems of diagnosis, differential diagnosis, and therapy are briefly mentioned.

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Fatal Peroral Iron Poisoning in a Young Woman

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During recent years a considerable number of severe or fatal accidental poisonings by iron salts has been reported [1 2 3 5, 6 7 8 12 13 14] In the USA approximately 2000 cases of iron intoxication are encountered yearly with a death rate of 45 % [4] In most cases children ingested tablets of ferrous salts, usually coloured or coated with chocolate. The exact dose of iron ingested could only be determined accurately in 36 % of a group of 172 children [4] The introduction of deferoxamine changed the treatment and improved the *fatal outcome of iron poisoning [10] The death rate is now lower than* 10% if proper therapy is started early enough. The normal low iron excretion in urine is increased by deferoxamine to levels exceeding one hundred times the normal values [10 15]

The majority fo cases with iron poisoning reported were in children and it may be of interest to report a case of suicidal iron intoxication in an adult with a fatal outcome

Case Report

A 23-year-old woman was admitted to the Psychiatric Department with the diagnosis of psychoneurosis. Her fiancé reported that he had found her in semicomatous state vomiting brown and black masses which were suggestive of gastric hemorrhage. Thereafter the *patient admitted to have ingested approximately 100 tablets 2 days previously* because of conflicts in her family On admission the patient was semicomatous and said with weak voice that she took two bottles of antianemic tablets about 32 h before The local physician reported later that he found two empty bottles of antianemic tablets, containing 50 mg iron each, in the patient room. The total dose taken was 10 g iron or 166 mg/kg body weight. The clinical findings were dark brown appearance of skin similar to sun tanning *Ptosis* of the lids was found, but the reflexes on examinations of the extremities were normal. Skin temperature was 36.5 °C, the blood pressure was not measurable, the pulse rate 130.

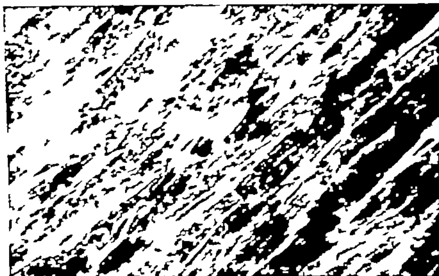


Fig 1 Myocardium. Sudan III Myocardial fibers with microscopic droplets of fat (ppearing as black spots).

Hb 72.2% (11.53 g %) RBC 3,830,000, CI 0.95, WBC 49,000, bands 1 %, segm. 81%, lymph. 12% monocyt. 6% Blood urea 84 mg %, Iron 480 μg %. Stool examination for occult blood positive.

After infusion of Ringer solution with Dexamethasone Rheomacrodex and antibiotic therapy the blood pressure rose to 70/60 mm Hg. The patient was then transferred to the Intensive Care Unit, where she died after admission.

Investigations (Intensive Care Unit) Blood urea 84 mg %, blood sugar 110 mg %. pH 7 133; 7 163; HCO_3 12.4 10.4 mEq/l total CO_2 13.5 11.3 mEq/l, physic. bound CO_2 1 12 0.9 mEq/l pCO_2 38.30 mm Hg, HCO_3/CO_2 1 10.9 1 11.6. Electrophoresis Alb. 62.5%, α-glob. 4.8%, α_2-glob. 6.4 %, β-glob. 8.8%, γ-glob. 18.5%.

Post mortem examination. The body is that of an adequately nourished woman. Both lungs show considerable degree of congestion and numerous hemorrhages especially on the anterior border I the pleural cavities approximately 100 ml of hemorrhagic fluid were found. The heart, pericardium and thymus ppeared normal and so did the gallbladder and pancreas. Lateral to both adrenals and under the capsules of the kidneys extensive hemorrhages were found. The kidneys ppear pale with normal ureters; the bladder which is also normal, contains no urine. The reproductive organs are normal as are the brain and meninges. The stomach shows an area of congestion containing numerous small hemorrhagic spots with some edema. *The stomach contains some millilitres of dark brown coffee-ground* material. In the duodenal area there are abundant hemorrhages. The small intestine contains dark tarry material especially in the ileum. The large intestine shows areas of congestion and swelling with hemorrhagic spots. The liver is pale, flabby greasy to touch, with areas of orange and yellow color The spleen is normal in size, weight and consistence.

Histology The heart shows fatty degeneration (fig. 1) as do also the epithelial cells of the tubules in the kidneys (fig 2). In the liver widespread and uniform fatty change was found. The droplets of fat in the cells are confluent, so that no protoplasm can be seen (fig 3)

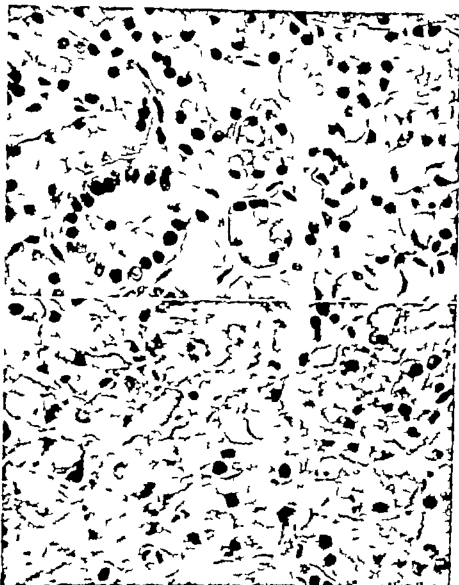


Fig. 2. Kidney. Haematoxyline-eosine. Numerous vacuolised tubular cells after extraction of fat.

Fig. 3. Liver. Haematoxyline-eosine. Heavy parenchymatous degeneration of liver cells and deposition of fat.

Staining with Sudan III shows the fatty degeneration to be of higher degree than expected by the hematoxyline-eosine method. The main lesions occur in the gastro-intestinal tract. In the stomach minimal changes were found, but in the duodenal mucosa severe necrosis of

the epithelium existed, so that no normal areas were encountered. I the deep layers the cell nuclei were colored pale or absent. The muscularis mucosae was edematous and thickened, the Brunner cells were weakly colored. It is of interest to not that in the tubules of kidneys and hepatic cells iron could not be demonstrated.

Discussion

REISSMAN *et al* [9] studied the mechanism of the toxic action of iron in animals. By stomach or duodenal tube or by enemas dissociable iron salts were given. The lethal dose in dogs and rabbits was found to be approximately 150 to 200 mg iron per kilogram body weight. In the majority of the animals no histologic changes were seen in the intestinal mucosa, but in some instances intestinal bleeding from congested capillaries occurred. In children who died from iron intoxication, necrosis of the intestinal mucosa was encountered, although in some cases minimal necrotic changes were found. REISSMAN estimates that the heavy intestinal necrosis in children is due to the corrosive effect of iron salts in substance while in the experiments with dogs and rabbits aqueous solutions have been used.

Poisoning with iron in adults is very rare. The patient reported passed at home of the three characteristic phases of iron intoxication 1 Acute gastro-intestinal symptoms, 2. relative wellbeing 3 fatal termination. She entered the hospital in the terminal phase of in toxication and died shortly after admission so that no therapy could be given. It appears that once the gastric mucosa is damaged, iron escapes from the gut into the tissues and leads to thrombosis of the submucosal veins. The numerous thromboses inhibit further iron absorption so that comparatively little iron is found in the liver and kidneys. It is difficult to come to another conclusion than that the extensive fatty changes in parenchymatous organs (liver heart, kidney) cause the rapid fatal outcome. The liver is not able to maintain its detoxicating function the heart becomes hemodynamically insufficient because of extensive fatty degeneration of its muscle fibers and the kidneys are damaged by the low blood pressure and fatty degeneration of tubular cells.

The cause of sudden death following relative wellbeing was obviously due to fatty degeneration of parenchymatous organs. Hyper ventilation and pronounced acidosis, suggesting a disturbance in oxydative metabolism, was present. In a recent investigation WITZ LEBEN and CHAFFEY [11] found extensive hepatic necrosis following

Prostatahypertrophie festgestellt wurde. Diese wurde zunächst konservativ behandelt als es im April 1965 jedoch zu einer akuten Harnverhaltung kam, wurde eine Ausschälung des Adenoms vorgenommen. Die histologische Untersuchung des Operationspräparates ergab keinen Hinweis auf Malignität. Nach der Operation war der Patient beschwerdefrei. Im Mai 1966 traten plötzlich am ganzen Körper blaue Flecken auf, ausserdem bemerkte der Patient eine ausgedehnte Blutung unter der Bindehaut. Einige Tage später kam es ausserdem zu einer Hämaturie, die so stark wurde dass der Patient schliesslich auf eine urologische Abteilung aufgenommen werden musst Hier ergab die i.v. Pyelographie und die zystoskopische Untersuchung keinen Hinweis auf eine lokale Blutungsursache Aus diesem Grund wurde nun eine Gerinnungsuntersuchung durchgeführt. Diese ergab eine schwere Gerinnungsstörung (siehe später) weshalb der Patient am 4.7 1966 zur weiteren Abklärung und Behandlung an die I. Medizinische Klinik verlegt wurde.

Die *klinische* Untersuchung bei der Aufnahme an der I Medizinischen Klinik ergab folgenden Befund Reduzierter Allgemeinzustand, blasse Hautfarbe, an der Haut keine Zeichen einer hämorrhagischen Diathese Herz und Lunge physikalisch unauffällig. Leber nicht vergrössert, Milz 1 QF unter dem Rippenbogen tastbar keine Lymphknotenvergrösserung, Nierenlager nicht klopfempfindlich. Rektale Untersuchung kein abnormer Befund an der Prostata erhebbar RR 170/90 mm Hg. Es bestand eine leichte Hämaturie

Laboratoriumsbefunde Senkung 70/46 mm, Serumweiss 6,7 g%, Albumin 3,1 g%, Globulin 3 6 g% Thymoltrübungsreaktion 1 E; Elektrophorese: Alpha$_1$- und Alpha$_2$-Globulinvermehrung. LAP 11 7 mE, Serumbilirubin 0,42 mg%, Bromsulphaleinretention nach 45 min 2%, Cholesterin 240 mg%, Harnstoff-Stickstoff 31 1 mg%, alkalische Phosphatase 3,1 m Mol E, saure Phosphatase 1,2 m Mol E. Blutbefund Ery 3,9 Mill, Hb 75%, FI 0,96 Leukozyten 6.550, Unsegmentierte 5%, Segmentierte 74%, Eosinophile 2%, Basophile 2%, Monozyten 1%, Lymphozyten 16%. Harnbefund Albumen positiv Saccharum negativ Urobilinogen 1 4 Sulkowitsch positiv; im Sediment reichlich Erythrozyten.

Röntgenuntersuchungen Thoraxröntgen Linksherz mit beginnender Mitralisation, vermehrte *Grundhelligkeit der Lungen. Kein Hinweis für blastomatöse Veränderungen.* Magen-Darm-Passage normaler Befund. i.v. Pyelographie Dürftige Ausscheidung des Kontrastmittels aus beiden Nieren mit vor allem rechts unvollständiger Darstellung des Hohlsystems. Unbehinderter Abfluss des Kontrastmittels in die nicht vollkommen zur Darstellung gebrachte Harnblase. Verdacht auf Anhebung des Blasenbodens bei Vergrösserung der Prostata. Skelettröntgen Hochgradige Spondylose mit multiplen Bandscheibendegenerationen und *Osteochondrose im Bereiche der Brustwirbelsäule. Mässige* Osteoporose.

Gerinnungsuntersuchungen

Bei der ersten Untersuchung des Patienten konnten folgende Befunde erhoben werden (Tab. I)

Als wesentliche pathologische Befunde wurde eine schwere *Fibrinogenopenie* bei gleichzeitiger Thrombopenie gefunden. Ferner bestand eine leichte Verminderung des Prothrombins und von Faktor V Es war auch eine leichte Verminderung von Faktor VIII anzunehmen, da der Thromboplastinbildungstest unter Verwendung von Patientenplasma leicht pathologisch war Eine vermehrt Fibrinolyse konnte mit dem Fibrinplattentest nicht nachgewiesen werden, die Euglobulinlysezeit war bei der ersten Untersuchung normal, bei späteren Untersuchungen konnte jedoch gelegentlich eine verkürzte Euglobulinlysezeit gefunden werden (Abb.2)

Das gleichzeitige Vorkommen einer Fibrinogenopenie und einer Thrombopenie deutete mit grosser Wahrscheinlichkeit auf eine Verbrauchskoagulopathie. Zur Verifizierung dieser Diagnose wurde aber noch die biologische Halbwertszeit von jodmarkiertem humanem Fibrinogen bestimmt. Wie zu erwarten, war die Halbwertszeit des Fibrinogens bei dem Patienten mit 12 h deutlich verkürzt (Abb.1) Die Halbwertszeit wurde noch kürzer (9 h)

Tabelle 1 Gerinnungsbefunde 24.6.1966

	Pat. H. L.	Normalwerte
Gerinnungszeit (LEE-WHITE) min	6	9
Blutungszeit (DUKE) min	7*	– 3
Thrombocytenzahl (BRECHER und CRONKITE)	90000	150000 250000
Thromboplastinzeit (QUICK), %	68	75–110
Prothrombin (F II) (DEUTSCH und SCHAUER) %	65	75–110
Proaccelerin (F V) (DEUTSCH und Schaden), %	67	75–110
Fibrinogen (CLAUSS) mg%	40	200–400
Partielle Thromboplastinzeit (LANGDELL) sec	77	50–70
Thromboplastinbildungstest (BIGGS)		
Pat.-Pl-Pat.-Serum	normal	
Pat.-Pl-Normalserum	leicht path.	
Serumprothrombin nach 1 h, %	30	< 10
Euglobulinlysiszeit h	über 8	über 12
Fibrinplatte	keine fibrinolytische Aktivität	

ja die Fibrinolyse durch tägliche orale Verabreichung von 10 g Epsilon-Aminocapronsäure gehemmt wurde. Wurde hingegen eine Dauerinfusion von Heparin verabreicht, kam es sofort zu einer deutlichen Verlängerung der Fibrinogenhalbwertszeit, wobei allerdings der Normalwert nicht erreicht wurde. Damit war erwiesen, dass die beobachteten Gerinnungsveränderungen, vor allem die Fibrinogenopenie und Thrombopenie, durch eine intravasculäre Gerinnung bedingt waren.

Beeinflussung der hämorrhagischen Diathese durch Heparin und weiterer Verlauf der Krankheit

Bei der Annahme einer Verbrauchskoagulopathie infolge intravasculärer Gerinnung war zu erwarten, dass eine Unterbrechung des intravasculären Gerinnungsvorganges durch Heparin die Gerinnungsstörung beseitigen würde. Wie aus Abb. 2 zu entnehmen ist, kam es tatsächlich schon 12 h nach Beginn der Heparintherapie (1000 E Heparin/Stunde als Dauerinfusion) zu einem deutlichen Anstieg des Fibrinogens, das am 4. Tag nach Therapiebeginn einen Höchstwert von 410 mg% erreichte. Die Thrombocyten stiegen ebenfalls an, aber erst nach einigen Tagen und nicht in demselben Ausmass wie das Fibrinogen. Gleichzeitig mit dem Anstieg des Fibrinogens und der Thrombocyten kam es zu einem Sistieren der Blutungsneigung.

Der gute therapeutische Erfolg konnte aber nur so lange aufrechterhalten werden, als Heparin kontinuierlich infundiert wurde. Als aus technischen Gründen die Therapie mit Depotheparin subkutan fortgeführt wurde, kam es wiederum zu einem Abfall des Fibrinogens, wenn auch nicht auf so tiefe Werte wie vor der Therapie.

Der weitere klinische Verlauf war gekennzeichnet durch einen rasch zunehmenden Kräfteverfall und Gewichtsverlust. Bei wiederholten rektalen Untersuchungen konnte schliesslich an der Prostata ein Tastbefund erhoben werden, der den Verdacht auf ein Prostatakarzinom nahelegte. Eine Probepunktion zur Verifizierung dieser Diagnose konnte

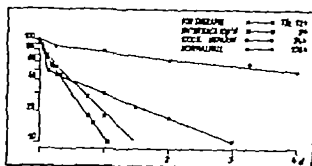


Abb. 1 Halbwertszeit von J131 Fibrinogen vor und während Therapie mit EACA 10 g tgl. bzw. Heparindauerinfusion (1000 E/h). Abszisse: Zeit in Tagen. Ordinate: Radioaktivität in % des 10-Minutenwertes.

Abb. 2. Einfluss der Heparintherapie auf Fibrinogenspiegel und Thrombozytenzahl.

—— Fibrinogen in mg%

●——● Thrombozytenzahl

wegen der hämorrhagischen Diathese aber nicht durchgeführt werden. Die saure Phosphatase war bei wiederholten Kontrollen nur grenzwertig. Der Patient verstarb plötzlich unter den Zeichen einer cerebralen Blutung am 3. 8. 1966.

Pathologisch anatomischer Befund

Bei der Obduktion (Sekt. Prot. Nr. 1146/66, Obd. Regele, Auftrag) des Patienten fand sich ein Prostatakarzinom mit Befall der regionären und paraaortalen Lymphknoten und aus-



Abb. 3. Fibrinthrombus in einem kleinen Lungengefäß. Mallory (× 300).

gedeckten Knochenmetastasen in der Wirbelsäule. In Leber und Lunge waren makroskopisch keine Metastasen nachweisbar. Als unmittelbare Todesursache war eine frische Massenblutung in die rechte Kleinhirnhemisphäre anzusehen. Weitere Zeichen einer schweren hämorrhagischen Diathese lagen in Form massiver Muskelblutungen im rechten Oberschenkel und verschiedenen subkutanen Hämatomen vornehmlich im Bereiche von Injektionsstellen vor.

Histologisch erwies sich das Prostatakarzinom als ein Adenokarzinom mit wechselndem Gehalt an doppelt-brechenden sudanophilen Tröpfchen im Plasma. Retroperitoneale Lymphknötchen waren ausgedehnt von analogen Karzinomformationen durchsetzt, Nekrosen oder Fibrinabscheidungen fehlten dabei. In den makroskopisch tumorfreien Lungen war im histologischen Schnitt eine geringgradige Lymphangiosis carcinomatosa, vor allem aber auch Tumorformationen in kleinsten Gefässen und interalveolaren Kapillaren zu sehen, vereinzelt und dabei unabhängig von den Tumorzellen gelegentlich auch Fibrinthromben (Abb. 3). Das Myocard ohne Auffälligkeiten, insbesondere die Gefässe mit freier Lichtung. Auch die Leber zeigte keine gröberen Veränderungen, in den Sinusoiden keine Fibrinthromben. Die Kupfferschen Sternzellen relativ gross und mit stark eosinophilem Plasma. Dieses zeigte bei der PAS-Reaktion eine deutliche Rotfärbung, die auch nach Diastasebehandlung bestehen blieb. Eisenfärbung negativ.

Der für diesen Fall wesentliche Befund war in den Nieren zu erheben. So gut wie alle Glomerula zeigten homogene eosinophile Ausgüsse in ihren Kapillarschlingen (Abb. 4). Diese gaben positive Fibrinfärbung und waren stets von der Kapillarwand abgrenzbar. Sie waren demnach als hyaline oder Fibrinthromben anzusprechen. Schlingennekrosen fanden sich nicht. Grössere und kleinere Rinden- und Markgefässe waren frei von Thromben.

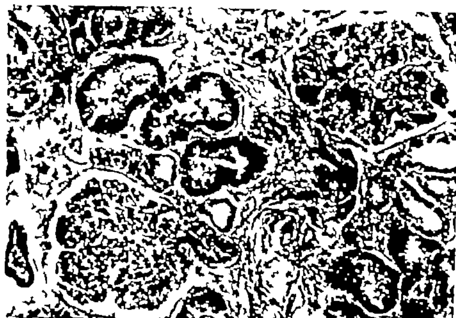


Abb 4 Hyaline Thromben in Schlingen von Nierenglomeruli. Mallory (× 400)

Diskussion

Die grosse Häufigkeit thromboembolischer Ereignisse bei Patienten mit malignen Tumoren ist dem Kliniker und Pathologen wohlbekannt. Eine wesentliche Ursache der Thromboseneigung beim Tumorkranken stellt sicherlich die Hyperkoagulabilität dar die vor allem durch Vermehrung oder Aktivitätssteigerung verschiedener Prokoagulantien [13] (Fibrinogen Faktor V und Faktor VIII) zustande kommt.

Klinisch äussert sich diese Thromboseneigung zumeist in Form von Makrothrombosen oder der sogenannten Thrombophlebitis migrans. Bei einer kleinen Zahl von Fällen führt die Hyperkoagulabilität aus bisher noch nicht genau bekannten Gründen jedoch zu einem anderen klinischen und pathologisch anatomischen Bild. Es kommt zur Bildung zahlreicher Fibrinthromben in den Kapillaren verschiedener Organe, ein Zustandsbild, das als diffuse intravaskuläre Gerinnung bezeichnet wird. Dadurch kommt es zum raschen Verbrauch von Gerinnungsfaktoren, vor allem des Fibrinogens und der Thrombozyten, so dass eine Gerinnungsstörung entsteht, die vor allem durch eine Fibrino-

genopenie und Thrombozytopenie gekennzeichnet ist und nach ihrer Entstehung als Verbrauchskoagulopathie [6] bezeichnet wird.

Der vorliegende Fall weist alle charakteristischen Befunde einer solchen Verbrauchskoagulopathie auf: Fibrinogenopenie, Thrombozytopenie, Verminderung von Faktor V und von Prothrombin. Auch die zeitweise nachgewiesene Fibrinolyse gehört zum Bild der Verbrauchskoagulopathie, da der Körper versucht, die Fibrinthromben durch Aktivierung der körpereigenen Fibrinolyse wieder zu beseitigen. Erwartungsgemäss war die Halbwertszeit des Fibrinogens deutlich verkürzt. Durch Gabe von Heparin konnte die Fibrinogenhalbwertszeit zumindest teilweise normalisiert werden, was als Beweis dafür anzusehen ist, dass die intravaskuläre Gerinnung tatsächlich pathogenetisch im Vordergrund stand. Gleichzeitig führte die Heparinbehandlung, solange sie intensiv durchgeführt wurde, zur Beseitigung der ursprünglich bestehenden Gerinnungsstörung und der Blutungsneigung.

Auch das morphologische Substrat dieses Zustandsbildes konnte in diesem Fall eindeutig nachgewiesen werden. Es fanden sich Fibrinthromben in grosser Zahl in den Kapillaren der Niere und im geringeren Ausmass auch in der Lunge. Der Nachweis von Fibrinthromben bei der Autopsie gelingt auch bei sicheren Fällen von Verbrauchskoagulopathie nur selten, was wahrscheinlich darauf zurückzuführen ist, dass die aktivierte Fibrinolyse und die postmortale Autolyse die vorhandenen Fibrinthromben rasch weiter verändern.

Verbrauchskoagulopathien können prinzipiell bei jedem malignen Tumor [4, 14, 17] auftreten. Allerdings neigen bestimmte Karzinome, wie die Karzinome des Gastrointestinaltraktes [1, 3, 5, 8], das Prostatakarzinom [5, 15, 16, 18, 19, 21] und das Pankreaskarzinom [5, 9] besonders häufig zu dieser Komplikation. Das Prostatakarzinom weist insofern eine Sonderstellung auf, als hier eine Defibrinierung nicht nur durch eine intravaskuläre Gerinnung, sondern auch durch eine Hyperfibrinolyse [10, 20] zustande kommen kann, was durch den reichlichen Gehalt des Prostatagewebes an fibrinolytischen Fermenten erklärlich ist. Es scheint aber, dass bei allen Fällen von Defibrinierungssyndrom beim Prostatakarzinom prinzipiell immer beide Vorgänge – nämlich Verbrauch und Hyperfibrinolyse – eine Rolle spielen, wobei einer der Mechanismen pathogenetisch im Vordergrund steht.

Wieso es gerade in bestimmten Karzinomfällen zur Verbrauchskoagulopathie kommt, ist noch nicht ausreichend geklärt. Es fällt aber auf, dass diese Fälle gewisse pathologisch-anatomische und

klinische Gemeinsamkeiten haben. Pathologisch-anatomisch handelt es sich zumeist um Adenokarzinome. Es war in den bisher beschriebenen Fällen immer eine hämatogene Metastasierung nachzuweisen, wobei sich Metastasen stets in Knochen oder Lunge oder in beiden fanden. Lebermetastasen wurden hingegen so gut wie immer vermißt. Klinisch waren diese Fälle im allgemeinen durch einen besonders foudroyanten Verlauf charakterisiert, der wahrscheinlich als Ausdruck des Einsetzens einer massiven hämatogenen Metastasierung anzusehen ist. Innerhalb kurzer Zeit könnten aus den zirkulierenden Tumorzellen beträchtliche Mengen an thromboplastischen Substanzen freigesetzt werden, wodurch die Clearancekapazität des RES und die körpereigenen Inhibitoren überspielt werden. Damit wären die Voraussetzungen für eine intravaskuläre Gerinnung gegeben.

Zusammenfassung

Es wird der Fall eines 71jährigen Patienten mit einem Prostatakarzinom beschrieben, bei dem es akut zum Auftreten einer Blutungsneigung kam. Als Ursache wurde eine Verbrauchskoagulopathie festgestellt. Durch Gabe von Heparin konnten die Gerinnungsstörung zeitweise beseitigt und die ursprünglich verkürzte Fibrinogenhalbwertszeit verlängert werden. Bei der Autopsie wurde ein Prostatakarzinom mit regionären Lymphknotenmetastasen und hämatogenen Metastasen in den Knochen und der Lunge gefunden. Histologisch konnten in der Niere und der Lunge Fibrinthromben nachgewiesen werden.

Summary

A 71-years-old patient with metastatic prostatic cancer suddenly developed severe hemorrhagic diathesis. Fibrinogenopenia, thrombocytopenia, reduction of the factors II, V and VIII and shortened half-life time of ^{131}J labeled fibrinogen were found. Diffuse intravascular clotting was suggested and heparin by continuous infusion was administered. This led to marked increase of fibrinogen and partial correction of the shortened half-life time of fibrinogen. At autopsy adenocarcinoma of the prostata with bone and lung metastases was found. Fibrin thrombi could be detected in the capillaries of the kidney and the lung.

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A Rapid Clinical Method for the Estimation of Activity of Aconitase in Blood

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The enzyme aconitase belongs to the class of hydrase enzymes which add water to organic compounds without splitting The reaction may involve an intermolecular oxydation reduction coupled with final hydration process. This is often a preliminary reaction step before starting biological oxydation.

The methods for the estimation of aconitase are based on the fact that it converts citrate to cis-aconitate which is further transformed into *dl*-isocitrate Citrate $\rightleftharpoons$ cis-aconitate $\rightleftharpoons$ *dl* isocitrate.

In a simple spectrophotometric method the light absorption due to cis-aconitate at 240 nm wave length is measured as an index of the enzyme activity *in presence of citrate or cis-aconitate as the substrate* [1] This procedure is unsatisfactory for direct estimation of enzyme activity in presence of haemoglobin or blood protein which has a high extinction coefficient at 240 nm wave length The enzyme activity may also be measured from the amount of citric acid produced from cis-aconitic acid by the enzymatic reaction and determined colorimetrically as a pentabromoacetone derivative [2] This method is accurate but not suitable for rapid clinical assay of blood aconitase.

While exploring the possibility of obtaining a reliable method *suitable for rapid evaluation of the status of aconitase in human blood* a simple method was evolved. The principle of this method is as follows The activity of the enzyme is determined colorimetrically by following the rate of reduction of the redox dye 2-6-dichlorophenol indophenol in presence of sodium citrate pig heart extract (isocitric dehydrogenase) nicotine adenine dinucleotide phosphate (NADP)

manganese chloride and phosphate buffer pH 7 4 The rate of reduction is followed in a Leitz photoelectric colorimeter at 610 nm wave length. A suitable substrate blank without citrate and NADP is run simultaneously to eliminate non-specific reduction of the dye This method is modified from the spectrophotometric method employed by BARBAN and SCHULZE [3] The unit of activity is taken as the rate of change of optical density per minute under the condition of the test.

Material and Method

Five ml of venous blood collected using heparin as the anticoagulant are used for colorimetric determination of activity of aconitase as detailed below The same sample is also analysed by standard spectrophotometric [4] method for the estimation of aconitase activity

Colorimetric method

Immediately after collection, the blood is transferred to an ice bath; 0.5 ml of whole blood is dissolved in 4.0 ml of ice cold distilled water and kept in the ice bath for 10 to 15 min when the sample ppears clear Then 0.5 ml of 0.2 M phosphate buffer pH 6.8, is mixed with the solution and preserved in the ice bath till the end of the assay

Into pyrex test tube (6″ × ¾″), the following reagents are added and maintained at 38° in water bath Phosphate buffer pH 7 4, 0.2 M 1.0 ml 0.06 M Sodium citrate 0.5 ml; distilled water 3.9 ml manganese chloride 0.001 M 0.1 ml.

A blank reaction mixture tube is also prepared as follows Phosphate buffer pH 7 4 0.2 M 1.0 ml distilled water 4.4 ml manganese chloride 0.001 M 0.1 ml.

Pig heart acetone powder extract in 0.1 M phosphate buffer of pH 7 4 containing 50–100 µg of protein/ml prepared and preserved at 4° C is used as source of isocitric dehydrogenase.

A NADP solution containing 2 mg of NADP (Sigma) per ml of distilled water is placed in test tube in ice bath.

A Leitz photoelectric colorimeter is adjusted to 100% transmission against distilled water at 610 nm wave length 0.1 ml of 2–6-dichlorophenol indophenol (0.035%) is taken in 1 cm³ cuvette. Pig heart extract 0 1 ml, NADP solution 0.1 ml and 0.2 ml of blood solution (1 in 10 dilution) are added in succession to reaction mixture tube maintained at 38 °C in water bath. The whole mixture is then poured into the cuvette containing the dye. This procedure ensures thorough mixing of the dye and the reactants. A stop watch is started after taking the initial (zero time) reading. Subsequent readings are taken at an interval of 1 min for 4 min. The above procedure is repeated with blank reaction mixture

The rate of change of optical density (O.D) is calculated for both the substrate and blank reaction mixtures from the linear part of the activity curve. The corrected value 'delta (Δ) is obtained by subtraction of the value for blank activity from that of substrate activity

All estimations are done in duplicate and the unit of enzyme activity is taken as the rate of change of O.D. per minute under the condition of the test.

The final enzyme activity is calculated as follows

Corrected 'delta	=	0.0047
PVC (packed cell volume)	=	32
Dilution factor	=	$\frac{6 \times 10}{0.2} = 300$

Table III. Units of aconitase activity in iron deficiency anaemia

Name	Hb g%	PCV %	Ret. %	WBC/mm³	Enzyme activity/ ml blood/min
S. D.	6.09	23	7 4	7 700	0.55
G R.	5.8	22	6.6	5,300	1 12
D. N	5.2	19	4.0	8,200	0.55
J N D.	5.2	23	0.9	7 100	0.79
R. B.	8.7	30	1.9	6,300	0.63
A. P	9.8	31	1 1	18,400	1 13
S. B.	5.5	22	1.5	6,800	1.27
P C.	6.38	26	1.8	7,500	0
B. J	6.58	24	5.5	11,900	0.65
H. S.	3.19	16	12.0	5,600	0 48

Values for activity of aconitase obtained colorimetrically in normal and iron deficiency subjects are shown in tables II and III

Comment

In the system employed the redox dye 2.6-dichlorophenol indophenol probably plays the following role Citrate → cis-aconitate → *dl*-isocitrate. *Dl* isocitrate + NADP → oxalo-succinate + NADPH. NADPH + oxydized dye → NADP + reduced dye. The reduced dye does not undergo spontaneous oxydation and the fall in optical density is used as a measure of the enzyme activity The dye 2.6-dichlorophenol indophenol has also been employed in enzymatic assay of oxydized and reduced forms of NADP and diaphorase in colorimetric studies [5 6] The reducing effects of the substrate blank solution is due to non-specific reducing substances likely to be present in blood lysates. The linearity curve shown in figure 1 is plotted after subtracting the blank value Slope of the line or *A* expressed as the unit of enzyme activity per minute, by both spectrophotometric and colorimetric methods, gave comparable results as shown in table I It would appear that for rapid clinical estimations, colorimetric method may be substituted for spectrophotometric method. This method is sensitive enough to detect differences in the enzyme activity of normal and anaemic subjects particularly those with iron deficiency anaemia [7]

Acknowledgement

Grateful thanks are due to K. K. Mathen, All India Institute of Hygiene and Public Health, for statistical help.

Summary

A simple colorimetric method has been standardised for the rapid determination of aconitase activity in whole blood by using redox dye 2-6-dichlorophenol indophenol in the presence nicotine adenine dinucleotide phosphate isocitric dehydrogenase citrate and phosphate buffer pH 7.4. Comparison of the data on the same sample obtained colorimetrically and spectrophotometrically showed no statistically significant difference. Evaluation of the enzyme activity in blood of normal and iron deficient subjects showed a deficiency of aconitase in the latter group.

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Book Reviews

R. Dybkaer und K. Jørgensen: Quantities and Units in Clinical Chemistry. Munksgaard, Copenhagen 1967

In den letzten Jahren hat sich die klinische Chemie immer mehr zu einem selbständigen Fach entwickelt. Eine Vielzahl von körpereigenen Stoffen werden mit immer komplizierteren Apparaten unter teils komplexen Versuchsbedingungen analysiert. Ein echter Fortschritt ist aber nur dann gewährleistet, wenn eine auf der ganzen Welt einheitliche Terminologie in bezug auf die analysierten Stoffe und Ausdrucksformen in bezug auf Grössen und Einheiten verwendet wird. Welche praktisch medizinischen Fortschritte die Wahl einer richtigen Einheit mit sich bringen kann, hat der Übergang von g/100 ml auf mAeq/Liter bei den Plasma-Elektrolyten gezeigt. Ähnliches muss auch auf allen anderen Bereichen angestrebt werden. Das Buch von Dybkaer und Jørgensen liefert nicht nur Ansätze zu einer solchen einheitlichen Terminologie der Mass-Einheiten, sondern legt ein weitgehend vollständig ausgearbeitetes System vor. Das Buch sollte von jedem gelesen werden, der sich mit wissenschaftlichen Fragen jeder Art auseinandersetzt. Nur dann ist zu hoffen, dass unverständliche und zweideutige Mass-Einheiten wie g% aus der Literatur verschwinden.

R. Richterich, *Bern*

R. Waitz: Actualités en transfusion sanguine. 2e cours européen de transfusion sanguine organisé par le Conseil de l'Europe, Strasbourg 1966. Masson, Paris 1967. 184 S., 34 Abb., 50 Tab. Preis 46 F

Seit einiger Zeit organisiert der Europarat alljährlich einen Kurs für Transfusionskunde, zu welchem die Mitgliederländer des Rates jeweils 1–2 jüngere Spezialisten dieses Fachgebietes delegieren. Der zweite Kurs fand vom 3.–14. Mai 1966 unter Leitung von Prof. R. Waitz im Blutspendezentrum Strassburg statt. Die praktischen Kursarbeiten wurden umrahmt von 11 Übersichtsreferaten, gehalten von den führenden Fachleuten Frankreichs. Der vorliegende Band vereinigt diese Referate und vermittelt dadurch einen ausgezeichneten Überblick, namentlich über die immunhämatologischen Aspekte der Bluttransfusion.

A. Hässig, *Bern*

Primates in Medicine. Vol. 1. First Holloman Symposium on *Primate Immunology and Molecular Genetics.* Ed. by C. H. Kratochvil. Karger, Basel/New York 1967. XVI + 99 p., 33 fig., 28 tab. Price: sFr./DM 16.50, US $ 4.00/30 s.

The book contains contributions from eight research groups which were presented at the Symposium held on September 28/30, 1966, at the *Aeromedical Research Laboratory of the Holloman Air Force Base,* New Mexico (USA). Simultaneously with the increase of knowledge on human immuno-genetic systems, the study of such systems in non-human primates is gaining growing interest. Six out of the eight studies are dealing with this subject. Moor-Jankowski and Wiener are giving a survey on human-type, simian-type and crossimmune-type of blood groups in primates. Masouredis *et al.* are presenting new data on the nature of Rh-antigens, obtained with the method of I 125 anti Rh_0 (D) uptake on the red cells. Wiener *et al.* are reporting on the complex relationship of the chimpanzee C-c-E-F system to the human Rh-Hr system. Poulik's paper is dealing with the structural similarities between immunoglobulins of human, chimpanzee and other non-human primates and Alepa is presenting evidence that chimpanzee sera contain at least four Gm- and two Inv-factors, known to be isoantigenic determinants of human γG-immunoglobulins. Finally, Goodman reports on his immunological investigations on proteins of primates

which – besides other interesting results – have revealed extensive transferrin polymorphisms in *Macaca mulatta*, chimpanzees and gorillas. Out of the two remaining studies, one by HOFFMAN and GOTTLIEB deals with hemoglobin variants of chimpanzees and gibbons, the other by KIMBALL, is an investigation on non-toxic immune-suppressive agents (performed not on primates but on mice) The Symposium has provided valuable informations on current researches in primate immunology and molecular genetics. Each of the studies gives lot of ideas and suggestions for further investigations which will also interest scientists working exclusively in human serology

R. BÜTLER, *Bern*

J GANCE Das «Banti-Syndrom» im Kindesalter VIII+133 S., 31 Abb., 14 Tab. Enke Stuttgart 1968. Preis DM 36.

Der Autor bemüht sich um eine Klärung des Begriffes Banti-Syndroms. Neben der Aufzählung symptomatischer Formen, insbesondere bei Pfortaderhypertonie hält er an der Existenz einer primären, essentiellen Form fest. Er beschreibt im Detail die klinischen Symptome und den Verlauf bei 15 Patienten, die er hier einreiht. Durch Bestimmen erythrocytärer leukocytärer und thrombocytärer Antikörper wird versucht, einen Beitrag zur Ätiologie des Syndroms zu liefern. Auch die Rolle der Milz im Sinne einer Dysplenie und die Zellphagocytose werden erörtert. Die spätere Mitbeteiligung der Leber im Sinn einer Zirrhose wird ebenfalls z.T auf Autoaggression zurückgeführt. Trotz den vielfältigen Untersuchungen bleiben viele Theorien in dieser anregenden Betrachtung eines schwierigen Problems spekulativ

M. VEST Basel

J M. COLLETTE, G JANTET and E. SCHOFFENIELS New Trends in Basic Lymphology Proceedings of Symposium held at Charleroi (Belgium) on 11 to 13 July 1966. Experientia, Supplementum 14 Birkhäuser Basel 1967 233 S. Preis F 48.

The papers on the rather young specialty of lymphology presented in the Symposium at Charleroi are reproduced in extenso in this volume. They concern the structure and ultrastructure of the lymphatic vessels, the properties and permeabilities of the lymphatic endothelium, the contractility of human lymphatics, the problem of lymphaticovenous communications, the effects of lymphatic stasis and the various methods for exploration of lymphatic structure and function.

There is no question that such booklet serves as valuable source of information to the basic scientist as well as the clinical investigator interested in lymphology

P FRICK, *Zürich*

Proceedings of the 10th Congress of the European Society of Haematology Strasbourg 1965. Part I Hauptvorträge. 312 Seiten. Preis sFr./DM 75.– Part II Kurzvorträge (2 Teile, 814 und 774 Seiten) Preis für beide Teile: sFr./DM 370.–

Traditionsgemäss erscheinen die Beiträge des 10. Kongresses der Europäischen Gesellschaft für Hämatologie in sorgfältig ausgestatteter Buchform. Der erste, bereits vor Jahresfrist veröffentlichte Teil enthält die Hauptreferate. Der Band ist für jeden hämatologisch orientierten Arzt und Biochemiker ein wertvolles Quellenwerk, welches auf knappem Raum neuere Aspekte in sorgfältig redigierten Übersichten über zentrale Probleme der Hämatologie zusammenfasst. Die Arbeiten von SADRON über Methoden der Untersuchung von Makromolekülen in Lösung und diejenige von KABAT über Struktur und Heterogenität von Antikörpern zeigen die Bedeutung und Fruchtbarkeit interdisziplinärer Zusammenarbeit zwischen Kliniker und Biochemiker Weitere, durchwegs originell dargestellte Beiträge behandeln die Geographie hämatologischer Erkrankungen, Probleme der Blutgerinnung, Fibrinolyse und Serologie, sowie neuere Aspekte der Leukämieforschung. Von besonderem praktischem Interesse ist die Arbeit von LAWKOWICZ und CZERSKI über die vergleichende Hämatologie verschiedener Tiergattungen, welche häufig für die experimentelle Forschung herangezogen werden.

or chronic renal insufficiency can be associated with a haemorrhagic disorder In the same year Salzman and Neri [5] and Castaldi *et al.* [6] investigated the dynamic platelet functions in the haemorrhagic diathesis of uraemia. This paper which is a continuation of that presented in 1966 describes the haemostatic mechanism in 19 cases of uraemia and deals mainly with platelet adhesiveness.

Material and Methods

Patients. The haemostatic mechanism was investigated in 19 uraemic patients who showed signs of a haemorrhagic disorder (tendency to epistaxis, spontaneous haematomas) at some stage of the disease. At the time of the examination 5 of these patients showed cutaneo-mucosal haemorrhages (purpura, haematomas, haemoptysis) All these patients had severe renal impairment with high blood urea levels (over 150 mg/100 ml) a markedly reduced glomerular filtration rate and isosthenuria. A few of them were treated by peritoneal dialysis and the others were on the waiting list for renal transplantation.

Methods. The platelet function was investigated using the following tests

1 Bleeding time Owren's method [7] was used. In our hands the normal values were between 3 and 7 min. The bleeding time is determined by the vasoconstriction platelet adhesiveness, aggregation and viscous metamorphosis.

2. Platelet adhesiveness We used Borchgrevink test which measures platelet adhesiveness *in vivo* [8]. Two platelet counts are made, one in venous blood and another in the blood flowing from a 1 cm long and 1 mm deep skin incision in the forearm (the same incision was also used for the bleeding time test by Owren's method) The difference between the two counts indicates the number of platelets adherent to the margins of the incision. The normal values represented as a percentage of the venous blood platelet count were, in our experience between 20% and 48%, with a average value of 34% [9].

3 The difference between the maximal amplitudes (ma) of the thromboelastographic curves in platelet-rich and platelet-poor plasma, expressed as ma1–ma2. The ma1–ma2 value depends on the thrombodynamic function of the platelets which determines their participation in syneresis. For normal platelet counts the ma1–ma2 value varies between 30 mm and 35 mm.

4 Clot retraction This test measures the retractive function of platelets.

5. Prothrombin consumption test Normal results are more than 25 sec. Results below this limit are abnormal and, if one can be sure that there is no deficiency of any plasma factor which takes part in the generation of the intrinsic activator they would indicate deficiency of platelet factor 3.

Results

Results are shown in table I The prothrombin consumption test was abnormal in 17 out of 19 patients (89.4%) with an average time of 20.3 sec which is below the normal limit of 25 sec. This was, therefore, an almost constant finding Borchgrevink's test showed decreased adhesiveness in 11 out of 19 patients (57.9 %) The average value of 18.8% was much below the normal value of 34% ± 7 %, and the differ

Table I. Chronic renal failure

Patients	No. of platelets per mm³ × 1000	Bleeding time min	TEG ma¹-ma² mm	Platelet adhesive ness in vivo %	Clot retrac tion	Prothrombin consumption index
1 F.L.	230	4	44	30	N	–
2 D. F	269	>20	39.5	20	N	21
3 A. A.	100	27	–	44	N	19
4 V. S.	298	15	29	8	N	–
5 D. C.	179	27	–	9	N	14
6 E. B.	90	–	–	22	N	19
7 P. S.	166	>20	45	0	N	15
8 J. T	138	4	40	17	N	21
9 D. C.	110	3	40	13	N	16
10 A. S.	115	>20	42	11	N	15
11 J. M.	381	–		22	N	–
12 S. P	310	5	–	0	N	31
13 L. B.	330	5	–	30	N	–
14 E. R.	340	15	34	15	N	30
15 J. O	315	4	36	8	N	23
16 J. V	283	3	28	43	N	21
17 O. D.	285	>15	46	36	N	19
18 C. C.	235	7	31	15	N	17
19 D. L.	263	>15	34.5	17	N	24
Mean	236	>12	31.2	18.8		20.3

ence was statistically significant. This difference is well represented in figure 1 where a comparison is made between normal adhesiveness and adhesiveness in uraemia. The bleeding time was prolonged (over 7 min) in 9 out of 17 cases (52.9%) The thromboelastographic index ma1–ma2, which represents the thrombodynamic function of the platelets, was abnormal in 2 cases. Clot retraction was always normal. The platelet count was within normal limits (average 236.000/mm against the normal average of 262.000/mm³) except in one case where it was below 100.000/mm³

Discussion

The above reported results show impairment of, at least, two platelet functions (1) deficiency in the plasma of platelet factor 3 and (2) deterioration in the dynamic function of platelet adhesiveness. In addition, CASTALDI has demonstrated impairment platelet aggrega

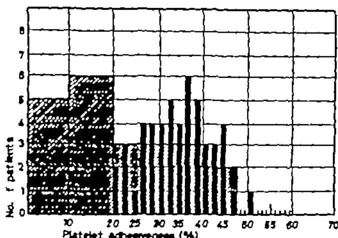


Fig 1 Comparison between the platelet adhesiveness in uraemia (obliquely striped zone) and that in 50 normal controls (solid columns)

tion. Thus, it is logical to accept that a true 'uraemic thrombopathy' does exist and is the cause of the haemorrhagic diathesis in chronic renal insufficiency. Platelet adhesiveness and aggregation become active during the first stage of haemostasis and their failure has a serious effect on early haemostasis. This is well demonstrated by the rapid commencement of bleeding and its long duration when the bleeding time test is performed. Although the bleeding time is not always prolonged, there is another characteristic feature of the abnormal test which is always present in cases of diminished adhesiveness. In normal subjects there is a time interval of 30–45 sec between the time the incision is made and the time when bleeding begins and forms a drop of blood sufficiently large to be drawn into a pipette. In cases of diminished adhesiveness this time interval is reduced to 5–10 sec. The possible cause of this phenomenon could be either absence of adhesive platelets to occlude bleeding vessels mechanically or the fact that abnormal platelets do not carry serotonin which contributes to the early vasoconstriction. In view of this observation, the axonal reflex postulated as the earliest haemostatic response appears to be much less important than dynamic platelet functions and, especially the first of these in chronological order i.e. adhesiveness. On the other hand, deficient generation of intrinsic thromboplastin impairs coagulation of the plasma which, in normal conditions, follows and reinforces the early phase of haemostasis.

Analysis of the correlation between the platelet adhesiveness, prothrombin consumption, thrombodynamic function and bleeding time in uraemia shows that

(a) Out of 13 uraemic patients with an abnormal prothrombin consumption the platelet adhesiveness was decreased in 8 and normal in 5 Two other patients with a normal prothrombin consumption showed decreased adhesiveness. Thus, these two tests of platelet function agreed in 8 and disagreed in 7 cases. Figure 2 shows a slight correlation between deficient prothrombin consumption and decreased adhesiveness but the number of tests is too small to attempt to express it in mathematical terms as an index of correlation (r) or covariation (p) The average values for both prothrombin consumption (20.3 sec) and adhesiveness (18.8 %) are below the normal limit.

(b) Comparison of the platelet adhesiveness with the thrombodynamic function shows that (1) Out of 9 cases with diminished adhesiveness the thrombodynamic function was normal in 8 and abnormal in 1 (2) out of 4 cases where adhesiveness was within normal limits the thrombodynamic function was normal in 3 and abnormal in 1 Thus, there is a considerable difference in the way these two platelet

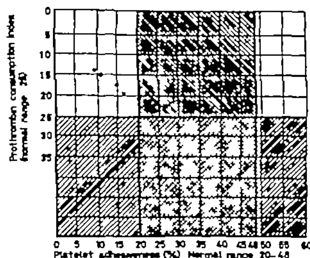


Fig 2. Correlation between platelet adhesiveness and the prothrombin consumption. The area divided into squares represents the normal range. Circles (O) indicate 'pairs of values' of adhesiveness and the prothrombin consumption. The cross (×) represents the average 'pair of values'

functions are affected adhesiveness is often impaired but the thrombodynamic function is usually normal.

(c) Finally the platelet adhesiveness and the bleeding time are compared in figure 3 which shows that (1) out of 11 cases of diminished adhesiveness the bleeding time was prolonged in 6 and normal in 5 (2) out of 6 cases with *normal* adhesiveness the bleeding time was normal in 3 and prolonged in the other 3 It is possible that decreased adhesiveness was compensated in some cases by vasoconstriction which participates in the haemostatic mechanism. The average adhesiveness was 18.8% and the average bleeding time 12 min There was therefore some correlation between decreased adhesiveness and a prolonged bleeding time.

On the other hand, the syneresis and clot retraction which are related to the so-called thrombodynamic function of the platelets were usually normal obviously these platelet functions are *independent of* the others.

Very little is known about the pathogenesis of the platelet defect in uraemia. We can only report that decreased adhesiveness and aggregation are not apparently related to low haematocrit values so fre

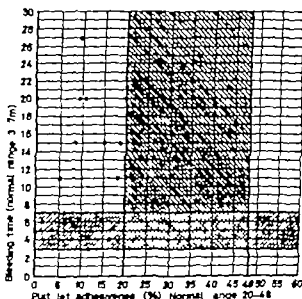


Fig 3. Correlation between platelet adhesiveness and the bleeding time. The area divided into squares represents the normal range Circles (o) indicate 'pairs of values of adhesiveness and the bleeding time. The cross (×) represents the average pair of values'

quently found in uraemia [10 11] and that HELLEM [12] has suggested that urea may interfere with platelet aggregation induced by ADP the meticulous investigations of SALZMAN and NERI [5] refute this hypothesis

SALZMAN and NERI [5] and CASTALDI *et al* [6] using different techniques, obtained results similar to ours. SALZMAN investigated platelet adhesiveness *in vitro* (by this technique normal values are between 25 % and 60 %) in 24 patients and found marked reduction to between 0 % and 19% in 21 of them. He found impaired adhesiveness in 7 out of 8 cases of gastrointestinal haemorrhage associated with uraemia. Like us, he observed some correlation but not full agreement, between deficient adhesiveness and reduced prothrombin consumption. In his opinion, deficient adhesiveness is an important factor in the uraemic haemorrhagic disorder and in the prolonged bleeding time.

CASTALDI *et al* [6] investigated the haemostatic mechanism in 19 cases of renal disease, 12 of which had haemorrhagic manifestations in 5 of the latter they found platelet adhesiveness *in vivo* of less than 20 (the normal range with their technique was between 20 % and 70 %) The bleeding time by Ivy s method, was more than 15 min in all patients who were bleeding, and in only one who had no clinical signs of a haemorrhagic disorder Platelet aggregation was also abnormal in the cases with a haemorrhagic tendency

Thus, both our investigations and those of other authors show a defect in the adhesiveness, in addition to the well known diminished prothrombin consumption, which is probably related to the haemorrhagic tendency and prolonged bleeding time in uraemic nephropathy It seems, therefore, that a deficiency of platelet adhesiveness together with a deficiency of platelet factor 3 may possible be an important factor in the uraemic haemorrhagic diathesis. The thrombodynamic function, on the other hand, is usually normal.

Conclusions

In chronic renal insufficiency a decreased prothrombin consumption is the most frequent finding which indicates a deficiency of platelet factor 3 There is frequently diminished platelet adhesiveness which explains the prolonged bleeding time in some cases. The bleeding time is often prolonged. The thrombodynamic function is usually normal.

Summary

The bleeding time, platelet adhesiveness *in vivo* thrombodynamic function of platelets, clot retraction and prothrombin consumption were investigated in 19 cases of chronic uraemia associated with a haemorrhagic diathesis at some stage of the disease. A decreased prothrombin consumption as a result of deficiency of platelet factor 3 was found in almost all the cases, as well as very frequently diminution in the platelet adhesiveness, and often a prolonged bleeding time. It is suggested that impaired platelet adhesiveness and deficiency of platelet factor 3 play an important role in the uraemic haemorrhagic disorder

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(Vorstand: Prof. Dr. H. Braunsteiner)

Zur Diagnose monozytärer Leukämien mit zytochemischen Methoden[1]

F. Schmalzl und H. Braunsteiner

Neuere zytochemische Untersuchungen über die Herkunft der normalen Blutmonozyten haben die Theorie der myelogenen Abstammung dieser Zellen bestätigt [4, 14, 30, 31]. Mit der Klärung dieses Problems eng verbunden, ist die seit Schilling und Naegeli diskutierte Frage der Monozytenleukämien [3, 8, 22, 26, 28, 35]. Dem vielfach als «typische Monozytenleukämie» bezeichneten Typ Schilling, für den eine unmittelbare retikulohistiozytäre Abstammung der leukämischen Zellen bisher angenommen wurde, steht die sogenannte «myeloische Monozytenleukämie» Typ Naegeli, gegenüber, deren Zellen von zahlreichen Autoren als Paramyeloblasten aufgefasst werden. Mischtypen [8] zwischen diesen Formen wurden beschrieben.

Diese Unterscheidungen wurden anhand panoptischer und phasenkontrastmikroskopischer Untersuchungsverfahren getroffen [3, 8, 22, 25, 28]. Mit der Anwendung zytochemischer Verfahren ist eine einfache und klare Differenzierung der Monozytenleukämie gegenüber anderen Hämoblastosen möglich geworden [4, 7, 11, 14, 17].

Material und Methodik

Der vorliegenden Arbeit liegen die Ergebnisse von Untersuchungen an 5 Fällen monozytärer Leukämien zugrunde. Es wurden Ausstriche von peripherem Blut und von Knochenmarkspunktionen verwendet. Die Resultate wurden zum Teil mehrfach kontrolliert.

[1] Diese Untersuchungen wurden mit Unterstützung des Fonds „Kampf dem Krebs" durchgeführt.

Zur Durchführung gelangten folgende zytochemische Reaktionen.

1. Unspezifische Esterase mit α-Naphthyl-Acetat als Substrat (α-N-E). Wir verwendeten hierbei die von LÖFFLER angegebene Methode, die wir gering modifizierten [16, 30].

2. Unspezifische Esterase mit Substraten der Naphthol-AS-Reihe (N-AS-E). Es sind dies unterschiedlich substituierte Derivate des β-Naphthols, wie Naphthol-AS-, Naphthol-AS-D- und Naphthol-AS-LC-Acetat (N-AS-A). Diese Substrate geben übereinstimmende Resultate. Für den Nachweis der Hemmbarkeit der N-AS-E wurde Natriumfluorid (NaF) in der Konzentration von 1,5 mg/ml dem Puffer zugegeben [6, 16].

3. Naphthylamidase: Wir wählen diese unspezifische Bezeichnung, um jene Fermente zu kennzeichnen, die bei zytochemischen Nachweisverfahren Amide des β-Naphthylamins mit L-Leucin, D,L-Alanin oder D,L-Methionin spalten [1, 27, 29, 32] und die mit der echten Leucinaminopeptidase nicht identisch sind [5]. Geringe, offenbar substratbedingte Unterschiede im Verteilungsmuster des Enzyms lassen sich bei Verwendung der verschiedenen Substrate feststellen. Wir verwendeten für die vorliegenden Untersuchungen L-Leucyl-β-Naphthylamid. HCl mit der etwas modifizierten Technik nach SCHMALZL und KATZENBERGER [29, 32].

4. Die Darstellung der Naphthol-AS-D-Chloracetatesterase (N-AS-D-Cl-E) erfolgte nach der von MOLONEY et al. [21] angegebenen Methode.

5. Saure Phosphatase wurde in Anlehnung an die Methode von BARKA und ANDERSON [2] nachgewiesen.

6. Bei der Darstellung der alkalischen Phosphatase [9] und

7. der Myeloperoxydase [10] folgten wir den Angaben von KAPLOW.

8. Die PAS-Färbung erfolgte nach HOTCHKISS [23], die

9. Sudanschwarz B-Färbung nach SHEEHAN and STOREY [33]. Kernfärbungen wurden entweder mit saurem Hämalaun nach MAYER oder mit der DNS-Färbung nach FEULGEN durchgeführt.

Ergebnisse

Die Ergebnisse unserer ferment- und bausteinzytochemischen Untersuchungen an normalen *Blut-* und *Knochenmarkausstrichen* stimmen mit den Ergebnissen anderer Autoren weitgehend überein [7, 11, 14, 16, 17, 21, 30]. In der Tabelle I sind die Befunde der verschiedenen zytochemischen Reaktionen an leukämischen Monozyten zusammengefasst.

Unter den aufgeführten Fermentnachweisen zeigten insbesondere die sogenannte unspezifische Esterase und die Naphthylamidase auffällige Reaktionsergebnisse in den leukämischen Zellen (Tab. I).

Die Aktivität der unspezifischen Esterase war sowohl mit dem Substrat α-Naphthyl-Acetat wie auch mit den Substraten der Naphthol-AS-Reihe sehr hoch. Die hohe Aktivität der N-AS-E wurde durch NaF (1,5 mg/ml) in den normalen und leukämischen Monozyten vollständig gehemmt [6, 30].

Die Naphthylamidase, nachgewiesen mit den Substraten L-Leucyl-β-Naphthylamid HCl, liess in allen 5 untersuchten Leukämien eine deutliche Aktivität erkennen.

Naphthol-AS-D-Chloroacetat-Esterase war bei Fall 1 in mässigem Ausmass in den leukämischen Zellen nachweisbar. In den 4 anderen

Fällen fanden sich nur gelegentlich Spuren dieses Fermentes in den monozytären Zellen.

Sudanschwarz B-positive Phospholipoide liessen sich in allen 5 Fällen in geringem Ausmass feststellen Peroxydase ebenfalls nur in geringem Ausmass, fand sich nur in einem Fall, die anderen enthielten kaum Peroxydaseaktivität in den leukämischen Zellen. Wir ziehen die Sudanschwarzfärbung der Peroxydasereaktion vor da die Sudanschwarzfärbung in jüngeren, weniger ausdifferenzierten Zellen der myeloischen Reihe die noch keine Peroxydase enthalten, bereits eine positive Reaktion gibt.

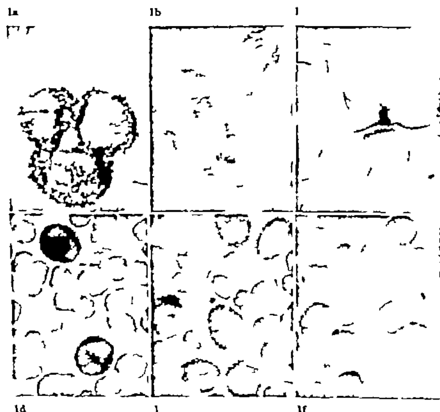


Abb. 1 Esteraseaktivität bei verschiedenen Leukämieformen. N-AS-E) b) und) In) Fermentaktivität durch 1,5 mg/ml NaF gehemmt. a-N-E d)) und f). Stark positiv reife Monozytenleukämie) und d). Weniger stark positiv Promonozyten-Leukämie b)) N-AS-E durch NaF in den leukämischen Zellen gehemmt (selber Fall wie) f deutliche a-N-E-Aktivität in Paramyeloblasten.) 1100 b)), d),) f) 1800 ×

Tabelle I. Angaben über weisses Blutbild, alkalische Neutrophilenphosphatase und Verhalten in den untersuchten Fällen von Monozytenleukämie. 0 keine ± unsichere

	Leukozyten pro mm³	Monozytoide Zellen in %	Index der alkalischen Leukozyten-phosphatase	α-Naphthyl-Acetat-Esterase
Normale Blutmonozyten				++ bis +++
Fall 1 Pat. R. 25 J	3 400	48%	108	++ bis +++
Fall 2: Pat. L. 60 J	8 100	38%	40	++ bis +++
Fall 3: Pat. B. 39 J	12 000	56%	96	++ bis +++
Fall 4 Pat. W. 44 J	187 000	93%	302	+ bis +++
Fall 5 Pat. A. 72 J	12 400	70%	43	+ bis +++

Der Gehalt an saurer Phosphatase war in allen Fällen beachtlich hoch. Alkalische Phosphatase wurde in den leukämischen Zellen nicht gefunden. Die Indices der alkalischen Phosphatase der Neutrophilen sind in Tabelle I zusammengefasst. Sie waren sehr unterschiedlich hoch, zum Teil wurden die hohen Werte durch bestehende Infekte bedingt.

PAS-positive Substanzen waren in allen Fällen, wenn auch in unterschiedlichem Ausmass feststellbar. In 4 Fällen war die Anfärbung diffus und mässig stark. Ein Fall zeigte neben einer diffusen zytoplasmatischen Anfärbung in zahlreichen Zellen Granula, die teils fein, teils grob waren und sich manchmal in grossen Plaques darstellten.

Auf Grund der Anfärbung bei den zytochemischen Reaktionen liessen sich die 5 Fälle in zwei Gruppen unterscheiden. Während Fall 1

ten der leukämischen Monozyten bei den verschiedenen zytochemischen Nachweisreaktio-
+ schwache ++ mäßig starke +++ sehr starke Reaktion

Naphthol-AS-Acetat Esterase	N-AS-E + 1,5 mg/ml NaF	Naphthylamidase	saure Phosphatase	alkalische Phosphatase	Naphthol-AS-D-Chloro-Acetat Esterase	Sudanschwarz B	PAS	Peroxydase
++ bis +++	± bis +	+ bis +++	++ bis +++	0	0 bis +	0 bis +	± bis +	0 bis +
++ bis +++	± bis +	+ bis +++	++ bis +++	0	0 bis ++	0 bis +	± bis +	0 bis +
++ bis +++	/	/	++ bis +++	0	0 bis +	0 bis +	± bis +	0 bis +
++ bis +++	±	+ bis ++	+ bis +++	0	0	0 bis +	0 bis +	0 bis +
+ bis +++	± bis +	0 bis ++	++ bis +++	0	0 bis +	0 bis +	0 bis +	0 bis +
+ bis +++	± bis +	0 bis ++	++	0	0 bis +	0 bis +	0 bis +	0

2 und 3 eine hohe Aktivität der unspezifischen Esterase in den leukämischen Monozyten und Monozytenvorstufen zeigen, wiesen die beiden anderen Fälle einen deutlich niedrigeren Gehalt auf. Dieser geringere Fermentgehalt kann mit einer gewissen Berechtigung als Zeichen einer mangelnden Ausdifferenzierung betrachtet werden. Tatsächlich waren in den beiden letzten Fällen zahlreiche unreife Zellen im peripheren Blut feststellbar

Um die Reaktionsintensität in den einzelnen Zellen einer Population besser zu erfassen, wurden jeweils 500 Zellen ausgezählt und je nach der gefundenen Reaktion in 4 Klassen eingeteilt (Tab. II) Besonders bei der Esterasereaktion zeigte sich deutlich, wie in den Fällen 1 2 und 3 die stärker positiven Formen überwiegen, während in den Fällen 4 und 5 die Mehrzahl der Zellen schwächer reagiert.

Tabelle II Reaktionsstärke in den leukämischen Monozyten bei verschiedenen baustein-
0 keine, + angedeutet bis schwache, ++ mäßig starke,

	α-Naphthyl-Acetat Esterase				Naphthol-AS-Acetat Esterase			
	0	+	++	+++	0	+	++	+++
Fall 1 Pat. R. 25 J	0	4	21	75	0	2	40	58
Fall 2 Pat. L. 60 J	0	5	27	68	0	8	30	62
Fall 3 Pat. B. 39 J	0	2	29	69	0	5	37	52
Fall 4 Pat. W. 44 J	0	12	38	50	0	42	46	12
Fall 5 Pat. A. 77 J	0	22	63	15	0	53	42	5

Diskussion

Normale Blutmonozyten sind auf Grund neuerer Untersuchungen in ihrer baustein- und enzymzytochemischen Ausstattung weitgehend charakterisiert. Ihre eindeutige Identifizierung in Ausstrichen von Blut und Knochenmark, sowie in Exsudaten und Infiltraten ist mit *verfeinerten zytochemischen Methoden möglich [4, 12, 14, 15, 29, 36]*. Die Theorie der Herkunft der Monozyten aus dem Knochenmark wurde mit diesen Methoden erneut untermauert, und die Abgrenzung *dieser Zellen gegenüber dem retikulohistiozytären System ermög*licht [4, 14, 30, 31].

Es erscheint nun naheliegend, diese zytochemischen Eigenschaften auch als diagnostische Kriterien für die Erkennung der Monozytenleukämie heranzuziehen *[11, 12, 14, 17, 20, 30]*. *In Tabelle I* sind die wesentlichsten kennzeichnenden Befunde zusammengefasst, die bei der zytochemischen Untersuchung normaler Blutmonozyten feststellbar sind. Ein entsprechendes Verhalten der leukämischen Zellen muss verlangt werden, um die Diagnose einer Monozytenleukämie oder falls es sich um jüngere Zellen mit noch nicht so ausgeprägter zytochemischer Ausstattung handelt, – einer Promonozytenleukämie zu stellen.

Unspezifische Esterase

Das Ausmass der mit den *Substraten* Naphthol-AS-Acetat und α-Naphthyl-Acetat feststellbaren Esteraseaktivität in den verschiedenen

und enzymzytochemischen Nachweisverfahren. Jeweils 500 Zellen wurden klassifiziert. +++ sehr starke Reaktion, × nicht durchgeführt.

Naphthol-AS-Acetat Esterase +1,5 mg/ml NaF				Naphthyl-amidase				Naphthol-AS-D-Chloracetat Esterase				Sudan-schwarz B			
0	+	++	+++	0	+	++	+++	0	+	++	+++	0	+	++	+++
20	80	0	0	10	16	22	52	12	70	18	0	96	4	0	0
×				×											
/	/	/	/	/	/	/	/	79	21	0	0	92	8	0	0
12	88	0	0	10	44	37	9	71	29	0	0	59	41	0	0
18	82	0	0	23	54	19	4	98	2	0	0	95	5	0	0
5	95	0	0	32	47	18	3	89	11	0	0	69	29	2	0

Zellformen stimmt nicht immer überein [14, 30]. Besonders ausgeprägt sind die Unterschiede bei verschiedenen hämatologischen Erkrankungen. So enthalten z. B. Plasmozytomzellen [18, 24], Erythroblasten bei erythrämischer Myelose [13, 30], bei Perniciosa [19] und bei toxischen Knochenmarkstörungen [30] und in Einzelfällen, was vielleicht zu wenig beachtet wird, auch Paramyeloblasten mehr α-Naphthylacetat-Esterase als Naphthol-AS-Acetat-Esterase.





Abb. 2. Naphthylamidasereaktion. a) stark positive Monozyten. b) Promonozytenleukämie mit z.T. schwach positiven Zellen. 1000

Ein spezifisches Kriterium zur Erkennung der Monozyten wurde mit der Feststellung der fast vollständigen NaF Hemmbarkeit der N AS-E in diesen Zellen und ihren Vorstufen gegeben [6] In anderen Zellen des Blutes und Knochenmarks, mit Ausnahme ausgereifter Megakaryozyten, ist diese Esterase kaum hemmbar [30] Für die Erkennung monozytärer Leukämien legen wir demzufolge mehr Wert auf die hohe durch NaF hemmbare Aktivität der N AS-E als auf eine hohe α-N E, die auch bei anderen Erkrankungen feststellbar ist. Die von uns bisher gesehenen Monozyten und Promonozytenleukämien liessen neben einer hohen α N E tatsächlich auch eine hohe durch NaF fast vollständig hemmbare, N AS-E erkennen. Im Gegensatz dazu wurde die mässig starke N AS-E leukämischer Promyelozyten und Plasmozytomzellen durch NaF kaum gehemmt, ebenso die geringe N AS-E der Myeloblasten- und Paramyeloblastenleukämie (Tab. I)

Naphthylamidase

Unter den Zellen des peripheren Blutes zeigen die Monozyten bei Verwendung des Substrates L Leucyl β-naphthylamid die stärkste zytochemisch nachweisbare Aktivität [32] Diese bleibt den Monozyten auch in Exsudaten, in Hautfensterpräparaten [29] und in Ergüssen [eigene Beobachtung] erhalten. Retikulumzellen enthalten viel Promyelozyten mässig viel und die übrigen Blut und Knochenmarkszellen enthalten wenig Naphthylamidase [1 27 32, eigene Beobachtungen]

Bei Monozytenleukämien liess sich das Ferment unter Verwendung von Leucin β-naphthylamid in unterschiedlicher Intensität in den leukämischen Zellen nachweisen im Durchschnitt war die Aktivität mässig hoch [1] In einigen Fällen fielen manche Zellformen durch ihren hohen Fermentgehalt auf. Sehr junge Zellformen – Monoblasten – enthielten ebenso wie *Myeloblasten keine Fermentaktivität.*

Naphthol AS-D-Chloracetat Esterase

Dieses Ferment ist, von den stark positiv reagierenden Gewebsmastzellen abgesehen für die Zellen der neutrophilen Reihe spezifisch *Basophile und Eosinophile enthalten in der Regel* keine entsprechende Fermentaktivität [16 30] Die in den Monozyten manchmal nach-



Abb. 3. Naphthol-AS-D-Chloracetat Esterase) nicht fermenthaltige leukämische Monozyten neben positivem neutrophilen Segmentkernigen; b) und) z.T deutlich positive leukämische Monozyten ( ) neben ngedeutet und schwach positiven (b) sowie stark positiver Promyelozyt (b) und Neutrophile ( ) b) und) selber Fall wie) und) der Abb. 1 1000 ×

weisbare geringe Aktivität wurde ebenso wie die Peroxydasereaktion der Monozyten, als Beweis für die enge genetische Beziehung der Monozyten zu den Zellen der neutrophilen Reihe angesehen [14]

Drei der beobachteten Monozytenleukämien (Nr 3 4 5) liessen keine Aktivität des Ferments in den Zellen erkennen. Der Fall 2 ent hielt nur in vereinzelten Zellen eine angedeutete Aktivität. Hingegen waren die leukämischen Zellen des Falles 1 z.T stark positiv unter schieden sich allerdings deutlich von der sehr ausgeprägten Reaktions intensität der wenigen vorhandenen reifen Promyelozyten, von denen sie sich im übrigen auch morphologisch völlig unterschieden Eine positive Korrelation zwischen dem Vorkommen reifer Promyelozyten im Blut und Knochenmark und stark N AS-D-E positiven Monozyten konnten wir nicht feststellen

Ähnlich den normalen Promyelozyten weisen auch die Zellen der Promyelozytenleukämien einen hohen Behalt an N AS-D-Cl E auf

Myeloblasten und Paramyeloblastenleukämien enthalten, wenn überhaupt eine sehr niedrige Aktivität des Fermentes.

Sudanschwarz B Färbung

Übereinstimmend zeigten in allen Fällen die leukämischen Zellen einen geringen oder keinen Gehalt an Sudanschwarz-anfärbbaren Substanzen. Bei den wenigen festgestellten stärker positiven Zellen dürfte es sich um Promyelozyten gehandelt haben Die leukämischen Monozyten stimmten in ihrem Verhalten mit normalen Blutmonozyten überein die in der Regel eine geringe Anfärbbarkeit mit Sudan schwarz B erkennen lassen Im Gegensatz dazu weisen Promyelozyten leukämien eine deutlich grössere Anzahl positiver Zellen und auch einen stärkeren Reaktionsgrad der Einzelzellen auf.

Für die Auswertung zytochemischer Reaktionen an hämatologi schen Präparaten wurde in Anlehnung an die score Technik zur Be-



Abb. 4 Sudanschwarz B-Färbung a) z. T. angedeutet positiv und b) völlig negativ leukämische Monozyten. Eine Anfärbung zeigen in a) ein eosinophiler Granulozyt und in b) ein neutrophiler Myelozyt. 1000 ×

urteilung der alkalischen Neutrophilenphosphatase mehrfach eine ähnliche Technik verwendet [20, 34]. Es scheint uns für diese Fälle allerdings zweckmäßiger, die Reaktionsintensität einer bestimmten Zellpopulation aufgeschlüsselt nach Reaktionsklassen in Prozenten anzugeben, wie wir es in Tab. II durchgeführt haben. Diese Darstellung gestattet eine bessere Beurteilung der gefundenen Aktivität und des Grades der Ausdifferenzierung der leukämischen Zellen unter der doch wahrscheinlichen Annahme, daß der Fermentgehalt der zunehmenden Differenzierung parallel geht. Bei reaktiven Monozyten im Rahmen von Stammzellenleukämien sind die Monozyten durch eine hohe, Stammzellen durch eine fehlende oder sehr geringe Esteraseaktivität gekennzeichnet. Es fehlen Zwischenstufen, die hingegen bei Monozytenleukämien vorhanden sind [14]. LEDER [14] entwickelte neuerdings die Theorie, die bisher als «jüngere» und deshalb fermentärmere Zellformen angesehenen leukämischen Zelltypen, z. B. «Myeloblasten», im Endstadium von Monozytenleukämien, seien das Resultat einer Entdifferenzierung der ursprünglich gewucherten Monozyten, etwa analog dem Vorgang der Entdifferenzierung bei Karzinomzellen. Ein Beweis für diese Ansicht steht noch aus.

Zusammenfassung

Auf Grund zytochemischer Untersuchungen läßt sich das Krankheitsbild der Monozytenleukämie eindeutig abgrenzen. Die gewucherten Zellen leiten sich von im Knochenmark gebildeten Blutmonozyten ab. Die Diagnose der Monozytenleukämie erfolgt auf Grund folgender zytochemischer Kriterien: 1. Der hohe Gehalt der leukämischen Zellen an unspezifischer Esterase bei der Verwendung von Naphthol-Acetaten als Substrat. Substrate der Naphthol-AS-Reihe sind besser geeignet als α-Naphthyl-Acetat, das erfahrungsgemäß auch in Paramyeloblasten eine positive Reaktion gibt. 2. Die Hemmbarkeit der starken Esteraseaktivität durch NaF (1,5 mg/ml) unter Verwendung der Substrate der Naphthol-AS-Reihe; 3. Eine mäßige bis starke Aktivität der Naphthylamidase in den leukämischen Zellen. 4. Die geringe Anfärbbarkeit mit Sudanschwarz B. 5. Eine in der Regel geringe Aktivität der Naphthol-AS-D-Chloracetat-Esterase in den leukämischen Monozyten.

Summary

Monocytic leukaemia can be clearly distinguished on the basis of cytochemical studies. The hyperplastic cells are derived from monocytes formed in the bone marrow. The following cytochemical criteria determine the diagnosis of monocytic leukaemia: (1) The high level of unspecific esterase in the leukaemic cells when naphthol acetates are used as substrate. Substrates of the naphthol-AS series are more suitable than alpha-naphthyl acetate, which occasionally gives a positive reaction also in paramyeloblasts. (2) Inhibition

of the marked esterase activity by NaF (1.5 mg/ml) when naphthol-AS substrates are used; (3) Moderate to marked naphthyl amidase activity in the leukaemic cells; (4) Poor staining with Sudan black B; (5) Usually limited naphthol-AS chloroacetat esterase ctivity in leukaemic monocytes.

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which with acute terminal blastic crises, chronic lymphoid leukemia (6) reticular (9) and lymphoid (3) disseminated sarcomatous diseases, Hodgkin's disease (12) and plasmocytoma (6). All these patients have been studied out of phases of hematologic or clinical remissions, generally in the first days after the admission in our Clinic. Moreover 6 patients with epithelial neoplasias and 9 with systemic lupus erythematosus have been studied.

Since the anti-I serum used in our researches has been obtained from a O-group patient, all the subjects included in this study were selected belonging to this erythrocyte group.

2) In vitro-interaction between Mycoplasmas and erythrocyte I antigen. The red cells of two normal O-group I-positive subjects have been suspended, after three preliminary washings, in sterile saline at 2% concentration, and incubated, respectively for 60 min to 22°C and for 24 h to 4°C with the following human Mycoplasma strains: Mycoplasma pneumoniae (Eaton agent) (FH strains); Mycoplasma fermentans (PG. 18); Mycoplasma hominis type 1 (PG 21) and type 2 (PG. 27), Mycoplasma orale (Patt strain). They have been grown (3–5 days at 37°C, for M. pneumoniae 10 days) in PPLO fluid medium [ALTUCCI *et al.*, 1966], then centrifuged 1 h at 12,000 rpm, and finally resuspended in saline at 100-fold concentration.

At the end of these two incubation periods, the red cell suspensions have been washed three times with sterile saline, resuspended in the same medium at 2% concentration, and were tested with the anti-I serum, adding 0.1 ml of the red cell suspension to 0.1 ml of the serum. The degree of the I-positivity of erythrocyte suspensions after the incubation with Mycoplasmas has been compared with that of the same suspensions before the addition of these micro-organisms.

3) Interaction between Mycoplasmas and I antigen in vivo. We have selected for this study the hamster infection with M. pneumoniae [7]. At first, we must remember that starting researches, performed for testing the behaviour of I antigen in the normal hamster red cells, have always shown (100% of the experiments) a marked I-positivity in the erythrocytes of these animals.

We have infected, in two different experiments, 4 hamsters, intranasal route (ml 0.1 × 2) previous Nembutal narcosis, with *M. pneumoniae* FH strain (10^{8} *CFU/ml*). Two animals were sacrified in the 10th day for reisolating on agar the infecting agent; and 2 respectively in 20th day for measuring the specific complement fixing antibodies.

For all the sacrified animals we have performed lung pathology evaluation, agar titration, at tenfold dilutions, of infecting agent, I-antigen research in the red cells, serum cold agglutinins [FEELER and HILLEMAN] and CF antibodies measurement [5, 1], reticulocyte count and Ht determination.

Results

All the red cells of patients suffering from leukemia or other malignancies have presented a strong I-positivity. We could not show any difference, with both types of incubation, between these erythrocytes and those from controls. As registered in table I, we have always

Coming the serum we have used from a O-group patient, we have examined, both for (1) and (2) all O-group red cells. It has been said [15] that Mycoplasmas are more active toward I antigen from A-group subjects: nevertheless, the same AA. admitted the possibility of an interaction (of biophysical and/or enzymatic order) [4] between Mycoplasmas and O-group cells.

The specificity of this phenomenon was demonstrated through the lack of agglutination of hamster red cells by both anti-A and anti-B serum.

Table I. Intensity of I-positivity of red blood cells of normal subjects, patients suffering from leukemia, malignancies and systemic *lupus erythematosus*

Groups	No. of cases	Anti-I serum agglutination	Intensity
Normal subjects	10	positive	+++
Acute leukemia	15	positive	+++
Chronic myelogenous leukemia	15	positive	+++
Chronic lymphoid leukemia	6	positive	+++
Reticulosarcoma	9	positive	+++
Lymphosarcoma	3	positive	+++
Hodgkin disease	12	positive	+++
Plasmocytoma	6	positive	+++
Epithelial neoplasias	5	positive	+++
Systemic *lupus erythematosus*	9	positive	+++

observed an I positivity with 3 + intensity comparable to that of controls. The same results were obtained in epithelial neoplasias and systemic *lupus erythematosus*.

The red blood cells of normal subjects, after the two different incubation periods with the Mycoplasmas, have shown neither a fall nor a loss of I positivity. The intensity of the agglutination by anti-I serum has been equal both for the red cells incubated and for those not incubated with Mycoplasmas (table II).

Although the infection by *M. pneumoniae* in hamster has been documented by the pathological findings in the lungs, by the isolation (in 10th day) of Mycoplasma, and by the content in CF antibodies (in 20th day) of the sera of the animals, we have not been able to show any change of the normally high I-positivity in the hamster red cells. The research of cold agglutinins has been negative; the reticulocyte count and the Ht value have not shown any difference in respect to the controls.

Discussion and Conclusions

Our results about the eventual loss of I antigen in red blood cells of patients affected by leukemia or other systemic and malignant conditions, and about the capacity of Mycoplasmas to inactivate I antigen in vitro or in vivo have been negative. They do not agree with the conclusions of McGinniss *et al.* [12], Schmidt *et al.* [14, 15], Barile *et al.* [4]. On the other hand, during the development of our work,

Table II. I-positivity of normal red blood cells before and after incubation with Mycoplasmas in vitro

Normal red blood cells samples examined	Mycoplasma strains tested	Red blood cells incubated at 22°C for 60 min and at 4°C for 24 h Intensity of I-positivity before incub.	after incub.
1	*M. pneumoniae* (FH)	+++	+++
	M. fermentans (PG. 18)	+++	+++
	M. hominis 1 (PG. 21)	+++	+++
	M. hominis 2 (PG. 27)	+++	+++
	M. orale (Patt)	+++	+++
2	*M. pneumoniae* (FH)	+++	+++
	M. fermentans (PG. 18)	+++	+++
	M. hominis 1 (PG. 21)	+++	+++
	M. hominis 2 (PG. 27)	+++	+++
	M. orale (Patt)	+++	+++

our data have been confirmed by the results of other authors [8, 13, 10].

It is not easy actually to find an explanation for these opposite results. From the technical point of view the most accurate works appear those of DUCOS *et al.* [8], SALMON *et al.* [13] and above all, by FEIZI and HARDISTY [10] who have performed a quantitative study of I antigen in red cells, and [at least FEIZI and HARDISTY] have tested both leukemic and control red cells against three different anti-I sera (whose two with high titer).

An interesting observation [10] is related to the different capacity of the various anti-I sera to detect a partial loss of I antigen (as, for example, that of some types of newborn red cells, I-cord). However it is not probable that this explanation may also partially justify the different results. On one hand it is clear that all the anti-I sera are able to show the complete loss of I antigen and/or its lack in the rare i cells in adult subjects [10]. On the other hand, the loss of I antigen in leukemic red cells [12, 14] has been observed with high concentrations of anti-I serum (1:2).

In our work, we could not perform a quantitative titration of anti-I reactivity of the various red cells examined, because of the low titer of the serum. Nevertheless, the intensity in the agglutination with 1:2 serum dilution clearly showed the normal presence of I antigen in the red cells. On the other hand, the technique followed by us is the same of the authors, who, on the contrary, have obtained positive results. The

Table III Behaviour of I-positivity related with other tests, in hamsters infected by *Mycoplasma pneumoniae*

Testing criteria	Normal hamsters sacrified	Hamsters infected with *M. pneumoniae* Sacrif. 10th day	Sacrif. 20th day
Lung pathology	absent	1) interstitial pneumonia 2) interstitial pneumonia	1) healing lesions 2) healing lesions
Agar titration of *M. pneumoniae* (CFU/ml)	n. d.	1) 2.10^7 $1.8.10^6$ 2) 1.10^7; $0.5.10^6$	1) $1.5.10^4$; 1.10^4 2) 0 2.10^4
I-positivity	+++	1) +++; +++ 2) +++; +++	1) +++;+++ 2) +++ +++
Cold agglutinins	n. d.	1) absent 2) absent	1) absent 2) absent
Specific CF antibodies	n. d.	1) <10 <10 2) 10; <10	1) 40 80 2) 40; 40
Reticulocytes, ‰	17–22‰	1) 20 20 2) 19 23	1) 19 18 2) 17; 18
Hematocrit, %	40–42	1) 38; 43 2) 40 43	1) 40; 43 2) 41 42

Expressed as the reciprocal of the highest serum dilution still able to show complete absence of hemolysis with 4–8 units of antigen and 2 full units of complement.

discrepancies with the results of these authors, obtained with *in vitro* incubation between Mycoplasmas and I positive red cells are – nevertheless – partial. They concern *M pneumoniae* (active, following American authors, in determining I negativity of erythrocytes) and *M fermentans* (only slightly active). Of other 3 strains tested by us, *M hominis* 1 has been inactive also in their experiments, *M hominis* 2 variably inactive or slightly active, *M orale* not tested. Contrarily to Schmidt *et al.* [14] we have not previously disrupted Mycoplasmas in these experiments by freezing and thawing twice. It is not easy to say how this difference may have contributed to give a different result.

More recently during the writing of our work, Feizi and Darrell [9] have reported that the treatment of OI red cells with two different strains of *M pneumoniae* did not affect the activity of any of three anti I sera tested either after overnight incubation at 4 C, or after incubation at 22° C. These data agree with our results.

Also the experiments concerning the behaviour of I antigen *in vivo* after a documented Mycoplasma infection have given conflicting results. On one hand, American authors [16 12] have recently noted

that the activity of the erythrocyte I agglutinogen was (only) transiently reduced following *M. pneumoniae* infection in 5 of 27 volunteers, without signs of hemolytic anemia. On the other hand, Feizi and Darrell [9] have found that the red cells from patients with *M. pneumoniae* infection (1st, 3rd, 7th week) reacted with the anti-I serum in a manner indistinguishable from the normal cells.

We have obtained similarly negative results in *M. pneumoniae* infection in the hamster.

However, it must be stressed that we cannot say, at present, whether so conflicting results, in the whole, may depend also from the eventually different antigenic distribution of various I positive red cells, and/or from the different source of anti I antibody in human sera tested (a natural agglutinin in I negative patient or a cold agglutinin from patients who had suffered or not for a *M. pneumoniae* infection). Also Smith *et al.* [16] admit that the greater sensitivity of the cold agglutinin anti I reagent than the iso-anti I reagent in detecting these antigenic changes may have been due to basic differences in molecular structure of anti I cold agglutinins found in cold agglutinin disease and iso anti-I cold agglutinins.

In conclusion, our data bring us to exclude both the changes in I positivity of leukemic red cells and the interaction between I-antigen and Mycoplasmas. It cannot be excluded that Mycoplasmas are the principal microbial candidates for the triggering of autoimmune conditions [3]. Nevertheless, our actual results bring us to consider with relative scepticism, also from this point of view, the possibility of an etiological relationship between Mycoplasmas and leukemia.

Summary

The red cells of patients affected by leukemia and other systemic or malignant diseases do not show the loss of I antigen after two different types of *in vitro* incubation with anti-I serum. Normal red cells, incubated *in vitro* with five different human Mycoplasma strains, retain their normal I-positivity.

M. pneumoniae infection in hamsters does not change the I-positivity of the red cells. The results of some authors, who showed the loss of I antigen in leukemic red cells and its interaction *in vitro* with Mycoplasmas, are discussed in relation with our negative results.

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separately were prepared for histological and chromosomal study and the remaining tissue was then transplanted into syngeneic hosts.

3. *Thymus from mice exposed to thigh-shielded irradiation.* Eight weeks following the first in a series of thigh-shielded irradiation, mice were killed and pieces of the thymus implant prepared for histological, chromosomal and transplantation study as mentioned above.

4. *Virus-induced lymphomas.* a) Four 7 and 10 weeks after inoculation of virus into the intrarenal implant in the thymectomized and irradiated recipients the animals were killed and pieces of the thymic implant prepared for histological, chromosomal and transplantation study as mentioned above.

b) In separate set of experiments, chromosomal analysis and histological examination were performed on 11 virus-induced disseminated lymphomas. No transplantation was performed on these animals since other experiments have shown that the disseminated lymphomas are readily transplantable [unpublished data].

Results

1. *Thymus from normal controls.* The chromosome pattern of thymus cells from 7 normal control mice of various ages is shown in table I. A metaphase of a normal thymus cell is shown in figure 1. Occasional cells contain only 39 chromosomes and a few cells have an extra chromosome. Although an attempt was made to count only cells that appeared intact, the possibility cannot be excluded that the missing and extra chromosomes are artefactual, due to excessive hypotonic treatment.

2. *Thymus from mice exposed to leukemogenic whole-body irradiation* (table II). Eight weeks after irradiation the thymic lobes were very small. Two were histologically classified as atrophic, indicating lymphocytic depletion [7]. The small size of these lobes made it dif

Table I. The chromosome pattern of normal untreated mice

Sex	Age weeks	No. cells with indicated chromosome number 39	40	41
F	6	1	33	
F	10		42	
F	21	1	36	1
M	20	2	36	1
M	20		41	
M	20	1	29	1
M	21	1	35	

Table II. The chromosome pattern and transplantation behaviour of thymus cells during the development of radiation-induced lymphomas

Weeks after X-ray	Mouse No.	Histol. classific.	Results of transpl.	No. cells with indicated chromosome number <38	38	39	40	41	42	>42	80	Structural chromosome aberrations	Final interpretation normal	equivocal	definitely abnormal
8	1	Atrophy				2	6	1					×		
	2	LCT				1	8	1					×		
	3	Normal			1	1	12					+		×	
	4	Normal				2	11						×		
	5	LCT			2	2	9	1				+		×	
	6	Atrophy		2	1		8		2	2		+		×	
14	1	LCT		1		1	24	2	1					×	
	2	LCT				2	17	2	1	4					×
	3	LCT				3	24	1	3						×
	4	IL	+	1	1	4	17		2	3		++			×
	5	IL		2	2	2	15	1						×	
	6	IL			2	7	18	4				+			×
20	1	LCT				1	11	2		5				×	
	2	IL			1	2	17	1						×	
	3	IL	+		4	7	4			2				×	
	4	IL	+	1	2	1	10			1				×	
	5	IL	+		1	4	13		1					×	
	6	IL			3		9		1	1		+		×	
	7	IL	+	1	2	2	28	2		1					×
	8	IL		1	2	2	12		1	4				×	
	9	IL	+	1	2	1	16	2	1	7	6				×
	10	IL		1	2	1	15	2						×	

LCT lymphoma confined to thymus, *IL* invasive lymphoma.

ficult to obtain good chromosome preparations and only relatively few metaphases could be examined. Occasional morphological aberrations were observed and in one atrophic lobe (No. 6) 7 of 15 cells showed aneuploidy. All of the implanted thymic lobes gave a negative transplantation test at 8 weeks after irradiation.

Fourteen weeks after irradiation all the lobes exhibited histological signs of tumour either as lymphomas confined to the thymic lobe or as invasive lymphoma. Only one however gave a positive take after transplantation. All these lobes showed numerical aberrations in chromosome pattern (fig 2) and some chromatid breaks were observed. Four of the 6 animals were classified as definitely abnormal.

At 20 weeks all lobes examined were histologically lymphomatous. Though the number of metaphases examined is small in some of the lymphomas it is obvious that all tumours analyzed at 20 weeks had substantial proportions of aneuploid cells. Two out of 10 lobes were classified as definitely abnormal. Six of 36 cells from one mouse (No 9) had chromosome numbers in the tetraploid range. Only occasional breaks were observed in these groups.

Five out of 10 lobes gave a positive transplantation test (table II). All of these were invasive lymphomas in a macroscopically and microscopically early stage. Mice No 2 exhibited slight dissemination to distant organs. Thus no correlation was found between specific morphological features and positive transplantation tests of the thymomas developing after irradiation. Nor was there a correlation between transplantation behaviour and the first appearance of numerical or morphological abnormalities in the chromosome patterns.

3. Thymus from mice exposed to thigh-shielded irradiation. Following irradiation with the thighs shielded the thymus undergoes characteristic histological alterations. Instead of the atrophy preceding the tumour development following leukemogenic whole body irradiation the thigh-shielded irradiation is attended by rapid recovery and hyperplasia of the thymus that becomes populated by small dense thymocytes [7]. Good spreading of the chromosomes is very difficult to obtain from these cells and a total of 40 acceptable metaphases could be obtained from 4 mice. The number of the chromosomes of these cells are presented in table III showing no major deviations from the mode of 40. No structural abnormalities were observed and the transplantation tests were all negative.

4. Virus-induced lymphomas (table IV). Four weeks after the inoculation of leukemogenic virus all of the three implants available for

Table III The chromosome pattern of thymus cells following thigh-shielded irradiation of the mice

No. cells with indicated chromosome number				Final interpretation		
38	39	40	41	normal	equivocal	definitely abnormal
2	4	35	1		×	

The table includes the chromosome counts of thymus cells from 4 mice

histologic examination were neoplastic. Two of these gave positive transplantation tests. Occasional aneuploid cells occurred but no convincing chromosomal aberrations were encountered. One of 4 were considered equivocal

Seven weeks after virus-inoculation all implants were neoplastic and exhibited histological evidence of invasive lymphoma although in an early stage of development. One of these, No 1 had about 30 of the cells in the hyperdiploid range Because of the very small size of this tumour few metaphases could be examined The other animals in these groups showed occasional loss of chromosomes but no major numerical or morphological abberrations occurred. Because of the small number of metaphases accessible to analysis the majority of these cases were classified as equivocal' All tumours gave positive takes after transplantation.

Ten weeks after virus-inoculation the tumours were histologically still in an early stage of development. Two of the lymphomas were still non-invasive. The chromosome counts indicate numerical aberrations in most of the cases, except for No 3 and possibly No. 2. One tumour No 5 had some missing chromosomes and one tetraploid cell and occasional chromatid breaks were observed. Four of the 5 tumours were classified as equivocal' Three implants, of which 2 were histologically classified as lymphoma confined to the implant, yielded positive transplantation takes. One invasive lymphoma with 5 out of 16 cells in the hyperdiploid range was negative after transplantation.

The chromosome pattern of virus-induced, disseminated lymphomas in the terminal stage of the disease is demonstrated in table V The counts indicate numerical abnormalities in all cases and 9 of the 11

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Double Blind Evaluation of the Haemostatic Effect of Adrenochrome Monosemicarbazone, Conjugated Oestrogens and Epsilonaminocaproic Acid after Adenotonsillectomy

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Evaluation of the haemostatic effect of agents designed to reduce blood loss is beset with numerous difficulties. Patients with a chronic haemorrhagic diathesis often bleed intermittently, haemorrhage ceasing or diminishing regardless of the drug administered. If the alleged beneficial effect of a haemostatic agent is tested when normal haemostasis is challenged, it must be ascertained that the severity of bleeding and underlying mechanism leading to it are similar in the different individuals. This latter requirement implies that traumatized persons or patients subjected to the multiple variants of surgery cannot be considered as valid test material for this purpose.

A more uniform challenge to haemostasis is a well standardized *bleeding time*, *but even in the best hands it is difficult to make a uniform* cut through the skin, due to the natural anatomical variations in vessel size. Because of other variables also, numerous cuts (e.g. 20 in the rabbit's ear) have to be made to ensure statistical validity [11]. *Reproducible results are also very difficult to obtain in man [15].*

In view of these drawbacks we chose adenotonsillectomy as a test situation wherein the mechanical trauma is fairly standard and large enough to permit pharmacologically induced reduction in blood-loss, if any, to be measured. Furthermore a large number of individuals can be included in the trial and as only healthy children are involved, the subjects can be considered as constituting a fairly homogenous group. The type of operation selected also offers the possibility of assessing blood-loss.

Three substances commercially available as haemostatic agents were evaluated in double blind trials using measured blood-loss as criterion in children subjected to adenotonsillectomy. The order of allocation of the test substance or placebo was randomly determined.

Methods

The experiments reported herein were performed in collaboration with the ENT department of the University Hospital of Louvain, Belgium. The subjects involved were all patients admitted to this department the night before operation and discharged 10 h after surgery. The technique used for tonsillectomy and adenoidectomy did not vary substantially among the different surgeons of the University department where all the operations were performed. Minor variations could not influence the results as control group was included in the series. No premedication was given; the child remained seated during the operation and was briefly anaesthetized with ethyl chloride. The adenoids were removed using the Vollmann curette and tonsillectomy performed by means of the Sluder tonsillotome. 80% of the children studied were subjected to both operations.

In the trial with adrenochrome monosemicarbazone, loss of blood through aspiration or ejection, was ascertained gravimetrically. Previously weighed dry gauze sponges, doctors' and nurses' blouses and bedlinen were used and the quantity of blood-loss determined by immediate re-weighing. The blood lost during the following 10 h was assessed using the same gravimetric method. The incidence of saliva and other ejected material in the weight difference can be disregarded because large control group underwent similar treatment.

In the trials with conjugated oestrogens and with epsilon-amino-caproic acid, total blood loss was determined by placing all the ejected blood, tampons, towels, doctors' and nurses' blouses and bedlinen used for one child in blood-loss monitor. The instrument used was the Perdometer (A.B. Lars Ljungberg, Stockholm). The blood containing materials were collected in tank containing 40 l of water and haemolysing agent. The blood was washed out by rotary washing mechanism and haemolysed. The blood-containing solution was filtered and pumped continuously through photometer that measured the light absorption due to haemoglobin content. Since this is proportional to the amount of blood present, the quantity of blood lost can be immediately ascertained by conversion table that takes into account the patient' preoperative haemoglobin value. The objection that swallowed blood is not taken into account can be disregarded because the same procedure was used for both the treated and control groups. The measurement of very small amounts in the Perdometer is not reliable below 10 ml of blood due to uneven distribution of fluids in the instrument (40 l) but abov this figure, the correlation with the measured value is good. The average error of the instrument in test determinations of known amounts of blood was 3% [14]. Haemoglobin was determined by the method of Drabkin.

Adrenochrome monosemicarbazone (1-methyl-2,3-dihydro-3-hydroxyindole-5,6-quinone) (Adrenoxyl® Labaz; Adrenosem® SE Massengill Co.) is one of the first derivates produced during the oxidation of epinephrine; the semicarbazone of adrenochrome is stable substance. Vials containing 5 mg active substance or placebo were prepared. The water soluble conjugated oestrogens (Premarin® Ayerst; Equigyne® Torrade) were used. Each vial contained 20 mg natural oestrogen in the dried state or placebo. The epsilonaminocaproic acid-vials (EACA) were Epsikapron® Kabi, Stockholm, containing 5 ml active substance at 40% (w/v).

The kind cooperation of the doctors and nurses of the ENT Department (Head: Prof. Dr. Fr. Charlier) is gratefully acknowledged.

Results

Adrenochrome

Vials with adrenochrome monosemicarbazone (5 mg) or placebo, identical in appearance, were prepared and numbered at random by the manufacturer and administered according to the double blind technique. The key indicating the content of each vial was kept in a sealed envelope which was not opened until the trial had been completed. A total number of 100 children took part in the trial of which 6 were eliminated at the end of the study because of incomplete data. Placebo was administered to 47 children and adrenochrome monosemicarbazone to the remaining 47 cases. Each patient received the night before amygdalectomy 5 mg adrenochrome intramuscularly and a second identical dose approximately half an hour before operation. This dose is considered to be high for children with an average weight of 22.3 kg

In this trial, blood loss was measured by a gravimetric method. The mean difference in weight of the linen before and after operation was 60 g (SD 21) in the adrenochrome-treated group and 58 g (SD 28) in the placebo-treated group. Both groups were similar in weight and height (table I)

Conjugated Oestrogens

This trial also was conducted strictly according to the double blind method, the sequence of substances given to the patients being determined by random allocation. All vials were similar in appearance. The content of one vial (?0 mg) was injected intramuscularly approximately half an hour before the operation. At all 200 children were involved in this trial upon completion of the study 27 cases (15 treated with conjugated oestrogens and 12 with placebo) had to be rejected for a variety of reasons likely to invalidate the results (loss of part of the linen, spitting of blood in the toilet or children leaving the ENT department before 6 p.m.) After disclosure of the code-key it appeared that, of the remaining 173 children, 85 received the test substance and 88 the placebo material.

The results of measured blood-loss are given in table I Both groups of patients were identical in weight and height, no difference in blood loss could be found.

Table I

	Monosemicarbazone of adrenochrome	Placebo
Number of children in the trial	47	47
Mean blood loss	58 g (SD 28)	60 g (SD 21)
Mean height	115 cm (SD 0.12)	112 cm (SD 0.18)
Mean weight	22.2 kg (SD 7.7)	22.2 kg (SD 9 7)
	Conjugated oestrogen	Placebo
Number of children in the trial	85	88
Mean blood-loss	85.4 ml (SD 57.8 ml)	89.8 (SD 37.0)
Mean height	117 cm (SD 16.1 cm)	115 (SD 15.7)
Mean weight	22.6 kg (SD 8.4)	21.2 kg (SD 8.08)
	EACA (2 g I.)	
Number of children in the trial	25	
Mean blood-loss	89.0 ml (SD 36.6)	
Mean height	121.5 cm (SD 15.06)	
Mean weight	23.9 kg (SD 6.8)	
	EACA	Placebo
	≤ 15 kg: 4 g I. > 15 < 30 kg 8 g I.v > 30 kg: 12 g I.	
Number of children in the trial	31	36
Mean blood-loss	60.3 ml (SD 17)	87.2 ml (SD 40.0)
Mean height	119 cm (SD 16.2)	125 cm (SD 15.4)
Mean weight	22.3 kg (SD 7.2)	22.6 kg (SD 8.3)

Epsilon-Amino-Caproic Acid

Vials containing 5 ml EACA at 40% were available. The content of one vial (2 g) was injected intravenously 30 to 60 min before operation in 25 children. There was no control group however the children involved in this trial were not preselected. The results of blood-loss are compared with the blood-loss obtained in the 88 control children, operated in identical circumstances. No difference was noted in measured blood-loss 89 0 ml (SD 36 6) in the treated group 89.8 ml (SD 37.0) in the control group of the previous trial.

As the possibility that the EACA dose was insufficient could not be excluded, it was decided to initiate a new trial whereby the EACA dose was increased and adapted to the weight of the child. The following doses of EACA were administered intravenously 30 to 60 min before the operation in children less than 15 kg 4 g EACA between

15 and 30 kg 8 g EACA over 30 kg 12 g EACA. A few instances of vomiting were observed after injection of the drug

As the EACA dose had to be adapted according to the weight of the child this part of our investigation was not a double blind but still a controlled not stratified study Each patient involved in the study was allocated to the EACA or placebo group according to serial numbering before the trial started each number was listed at random in one of the two groups. As a significant difference in blood loss was observed after treatment of 67 children (31 in the EACA group, 36 in the control group) this trial was not continued with the remaining 33 of the 100 children initially envisaged Both groups were similar with respect to weight and height. The blood-loss in the EACA treated group was 60.3 ml (SD 17) and in the control group 87.2 ml (SD 40 0 ml) this difference is highly significant ($P < 0\ 001$)

Discussion

The alleged haemostatic effect of conjugated oestrogens is based on the work of SCHIFF and BURN [17] These authors have demonstrated a progressive change from the sol to the gel state of mucopolysaccharides in the ground substance around small vessels and in the arterial walls. This polymerization of the mucopolysaccharides was observed in hamsters, monkeys and men following intravenous administration of conjugated oestrogens.

Detailed reports concerning the impact of conjugated oestrogens on blood coagulation are rare and often conflicting An elevation of fibrinogen [12] prothrombin and factor V and a decrease of antithrombin [6] have been described but these findings could not be confirmed by other investigators [7 9] It is possible however that capillary oozing could be shortened by action of oestrogens at the vascular or extravascular level without being influenced by the intravascular coagulation factors. An attentive study of the clinical reports is rather disappointing as the effect of conjugated oestrogens on haemostasis is based on clinical judgment only except in two trials conducted according to the double blind method which however were both negative [7 19]

It is beyond doubt that epinephrine from which monosemicarbazone of adrenochrome is derived has a vasoconstrictor effect on the microcirculation. The direct relationship between the decreased flow

of blood within these vessels and its alleged function in lessening blood loss is less clear As epinephrine is readily oxidized to adrenochrome which itself is also unstable, a more suitable agent for clinical use was developed the monosemicarbazone of adrenochrome which is said to be stable when administered by mouth or by injection [4]

Numerous studies have been published on monosemicarbazone of adrenochrome claiming its increased capillary resistance and usefulness in reducing haemorrhage after oral or parenteral administration Unfortunately the enthusiastic clinical reports on its haemostatic role in man originate from clinical impressions and/or uncontrolled clinical trials. The only reported double blind clinical trial failed to confirm previous favourable reports in this trial the observation of the duration of bleeding after dental extraction was made by the patient himself, thus exposing the evaluation method to criticism because of the ill-defined subjective criterion [5] In another controlled trial the same drug had no statistically significant effect on capillary resistance as measured by a negative pressure method. No apparent difference in the incidence of post-operative hyphaema in a series of forty pairs of patients, one of each pair receiving monosemicarbazone of adrenochrome by mouth, was observed [18]

Different fibrinolysis inhibitors are commercially available, some of them being extracted from tissue. The polypeptide isolated from bovine lung (Trasylol® Bayer) or pancreas (Iniprol® Choay) are inhibitors of trypsin, plasmin and plasminogen activator Synthetic fibrinolysis inhibitors are the aminoacids epsilon amino-caproic acid (EACA) Transamino-methyl-cyclohexane-carboxylic acid (AMCHA or tranexamic acid cyclocapron) and para-aminomethyl benzoic acid (PAMBA)

It has been demonstrated beyond reasonable doubt in controlled studies that synthetic fibrinolysis inhibitors can diminish bleeding from areas with increased local fibrinolytic activity as for instance in the urinary tract [1 8, 16] and uterus [10 15 20] The effective oral dose required is approximately 400 mg/kg EACA or between 60–90 mg/kg AMCHA.

Activators of the fibrinolytic system are present in almost all tissues as shown by the experiments of Astrup and Permin [3] After adenotonsillectomy haemorrhage at the adenoid and tonsil bed may be sustained because of the local activity of tissue activators, premature dissolution of local clots prolonging bleeding or provoking renewed blood-loss. It is a fair assumption to relate the haemostatic value of

15 and 30 kg 8 g EACA over 30 kg 12 g EACA. A few instances of vomiting were observed after injection of the drug

As the EACA dose had to be adapted according to the weight of the child this part of our investigation was not a double blind but still a controlled not stratified study Each patient involved in the study was allocated to the EACA or placebo group according to serial numbering before the trial started each number was listed at random in one of the two groups. As a significant difference in blood loss was observed after treatment of 67 children (31 in the EACA group, 36 in the control group) this trial was not continued with the remaining 33 of the 100 children initially envisaged. Both groups were similar with respect to weight and height. The blood-loss in the EACA treated group was 60.3 ml (SD 17) and in the control group 87.2 ml (SD 40 0 ml) this difference is highly significant ($P < 0\ 001$)

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Reticulohistiocytic Origin of the Blood Basophil in Human Marrow

E. M. SCHLEICHER

Since EHRLICH [1] described two types of mast cells the Blutmastzelle and the Gewebsmastzelle their origin and histochemistry have been studied Because of the structural, chemical, and functional similarities between the cells SELYE [2] states the blood basophil represents the circulating form of the tissue mast cell system However LENNERT and SCHUBERT [3] BRAUNSTEINER [4] BRAUNSTEINER and PAKESCH [5] SHELLY and JUHLIN [6] and WINQVIST [7] showed structural differences between the blood basophil and the tissue mast cell While the genesis of the blood basophil has remained conjectural UNDRITZ [8] derives the cell from a Basophiloblast

It is the purpose of this communication to present morphological evidence of a direct reticulohistiocytic origin of the blood basophil in human bone marrow For this study Hodgkin s disease was found to be particularly appropriate. Frequently the basophils are increased in the circulating blood and the bone marrow in this condition besides, the genesis of the blood basophil could be more easily studied than in leukemic marrows containing large quantities of undifferentiated and pleomorphic cells.

Material and Method

The cell studies were made on male 28 years old, who had generalized Hodgkin disease The peripheral blood basophils ranged from 8 to 14 determined by the method of SCHLEICHER [9] From the body of the sternum 'marrow units (Markbröckel) were aspirated The 'marrow unit imprint technique was employed [10, 11]. The preparations were tested by dual staining procedure namely Wright blood stain with Toluidin blue 0.5 aqueous

solution superimposed. The latter forms chemical union with the acid mucopolysaccharides in the granules giving them the specific metachromatic ppearance.



Fig 1 Reticulohistiocytic syncytium. ( ) Primitive reticulolymphoid cell (kleine lymphoide Retikulumzelle of Rosen) (b) Early developmental stage of the blood basophil. ( ) Advanced developmental stage of the blood basophil.

Fig 2. ( ) Histiocyte. (b) Variants of developmental stages of the blood basophil. Note the cytoplasmic tail' containing various amounts of granules of different size and degree of metachromasia.

Results

In the reticulohistiocytic syncytium (fig. 1) the earliest recognizable developmental stage of the blood basophil is a small reticulolymphoid cell 'kleine lymphoide Retikulumzelle' of ROHR (fig. 1a). The nucleus is round and often slightly notched and more or less eccentrically located. It has a reddish-purple color and several various sized bluish purple nucleoli. The narrow cytoplasm ranges from light blue to colorless and may contain small metachromatic granules. The cell boundary is demarcated by a condensation of the cytoplasm. This primitive cell differentiates within the syncytium into a blood basophil without going through a blast stage (fig. 1b, c). One to several basophils in various maturation stages may be present in a syncytium. The cells retain a histiocytic nucleus which becomes indented, lobulated and more or less overlayed by granules of different size and degrees of metachromasia. Various developmental stages are presented in fig. 2b. The pear-shape results from a cytoplasmic tail in which the granules tend to concentrate. This morphological feature appears to be characteristic of the developing basophil. According to these morphologic findings it is inferred that the blood basophil is a histiocyte.

Summary

The genesis of the blood basophil in human bone marrow as seen in marrow unit imprint preparations is presented. A direct origin from reticulohistiocytic syncytia could be demonstrated. The progenitor of the blood basophil is a small reticulolymphoid cell 'kleine lymphoide Retikulumzelle' of ROHR. It is inferred that the blood basophil is a histiocyte.

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In the reticulohistiocytic syncytium (fig. 1) the earliest recognizable developmental stage of the blood basophil is a small reticulolymphoid cell 'kleine lymphoide Retikulumzelle' of Rohr (fig. 1a). The nucleus is round and often slightly notched and more or less eccentrically located. It has a reddish-purple color and several various sized bluish purple nucleoli. The narrow cytoplasm ranges from light blue to colorless and may contain small metachromatic granules. The cell boundary *is demarcated by a condensation of the cytoplasm.* This primitive cell differentiates within the syncytium into a blood basophil without going through a blast stage (fig. 1b, c). One to several basophils in various maturation stages may be present in a syncytium. The cells retain a histiocytic nucleus which becomes indented, lobulated and more or less overlayed by granules of different size and degrees of metachromasia. Various developmental stages are presented in fig. 2b. The pear-shape results from a 'cytoplasmic tail' in which the granules tend to concentrate. This morphological feature appears to be characteristic of the developing basophil. According to these morphologic findings it is inferred that the blood basophil is a histiocyte.

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Neonatal Hemolysis Due to a Transient Severity of Inherited Pyruvate Kinase Deficiency[1]

M. Bossu, M. Dachà and G. Fornaini

In recent years many works have shown the high occurrence and severity of the hemolytic diseases in the newborn period. In many cases, such as those due to an isoimmunization to a bacterial and viral infections, to genetically determined metabolic deficiencies, to hemoglobinopathies, to morphological modifications of the cell the etiology and pathogenesis are fairly known. In other cases, the causes of the hemolytic episode are unknown. The latter forms may be ascribed to some morphological or functional transient disturbances of the cell.

However it might be that a transient state of deficiency is responsible for the hemolytic episode also in the subjects bearing an inherited defect and particularly in those cases in which some inborn errors of erythrocyte metabolism are present. Such a possibility is confirmed by the present case of hemolytic disease in a newborn infant being heterozygous for pyruvate kinase deficiency. In this subject the hemolytic state is determined by a transient severity of the genetic deficiency.

Case Report

M. P. Propositus. At 4 days age the patient colouring became pallid and he demonstrated icterus, vomiting and slight splenomegaly. Examination of peripheral blood provides the

The following abbreviations have been used: *G-6-PD* glucose-6-phosphate dehydrogenase; *6-P-GD* phosphogluconate dehydrogenase; *PK* pyruvate kinase; *G-6-P* glucose-6-phosphate; *6-P-G* 6-phosphogluconate; *GSH* reduced glutathione; *APH* acetyl-phenylhydrazine; *ATP* adenosine 5-triphosphate; *RBC* red blood cells; *WBC* White blood cells; *WEP* Whole erythrocyte population.

[1] The present work has been carried out with CNR founds.

following data: RBC 2,600,000, Hb 11‰, reticulocytes 4‰, slight aniso-poikilocytosis, small number of macrocytes and normoblasts, no spherocytes, WBC 7,200, serum bilirubine 16.8‰. Coombs test negative. The red cell osmotic fragility was normal before incubation and markedly increased after incubation at 37°C for 24 h. The autohemolysis was not corrected by adding glucose, and slightly decreased by adding glucose and ATP. The subject was not transfused and was treated with cortisone and albumin.

24 h after crisis RBC 2,600,000, reticulocytes 7‰, bilirubine 16.6‰; 48 h after crisis RBC 2,800,000, reticulocytes 9‰, bilirubine 15.4‰; 5 days after crisis RBC 2,800,000, reticulocytes 14‰, bilirubine 12.4 ‰; 10 days after crisis both icterus and splenomegaly disappeared and the hand-feeding was well accepted. RBC 3,300,000, reticulocytes 8‰, bilirubine 9.3‰; 20 days after crisis RBC 3,900,000, reticulocytes 3‰, bilirubine 5.8‰; 30 days after crisis RBC 4,200,000, reticulocytes 2‰, bilirubine 4.1 ‰.

The examinations of the subjects used as controls were normal, for the referred experiments, from a pathological point of view or from hematic tests.

Researches were always been carried out parallely on the pathological subject and on controls bearing approximately the same age.

Materials and Methods

Blood was collected with siliconated syringe and heparin added. After centrifugation the plasma and buffy coat were removed by suction and the red cells washed three times with isotonic buffered KCl solution at pH 7.4. The packed cell volume of these suspensions was determined by centrifugation at 3,000 rpm for 60 min in a hematocrit tube. The hemolysates were prepared by treating washed cells with two volumes of cold distilled water. The ghosts were then removed by centrifugation at 30,000 × g for 30 min and the supernatant collected. All procedures were carried out at +2°C.

Young and old erythrocytes were prepared from resuspended washed cells according to MARKS and JOHNSON's method [1], which is based on the principle that young erythrocytes are more resistant to hypotonic hemolysis than are older erythrocytes.

The glucose utilization by intact erythrocytes was determined according to previously described procedure [2] and the reduced glutathione stability according to BEUTLER [3] in presence of 5×10^{-3} M glucose.

G-6-PD and 6-P-GD determinations were performed according to KORNBERG and HORECKER [4] and HORECKER and SMYRNIOTIS [5] following GLOCK and MCLEAN's suggestions [6] to remove the 6-P-GD interference on 6-P-G derived from G-6-P. Pyruvate kinase determinations were performed according to BÜCHER and PFLEIDERER [7] and Triose P isomerase determinations according to BEISENHERZ [8].

Glucose was determined with glucose oxidase [9]; lactic acid according to BARKER [10]; GSH according to GRUNERT and PHILLIPS [11]; hemoglobin concentration with DRABKIN's method [12].

In experiments of erythrocyte separation on the basis of different osmotic resistance 'young-cells' are those unlysed after hemolysis with 0,50% NaCl for 15 min at +2°C; old cells are those hemolysed with 0,46% NaCl under the same conditions. The number of cells was determined by hemolysis achieved by a given concentration of NaCl, taking the extent of hemolysis in a water tube as equal to 100%.

In the GSH content is expressed in mg/100 ml of packed erythrocytes. The values of glucose uptake and the level of enzyme activities are referred as µmoles of metabolized substrates per hour and per ml of packed erythrocytes.

Substrates, enzymes and coenzymes were products of Sigma and Biochemica Boehringer other chemicals of Merck. Spectrophotometric determinations were carried out with Optica

CF4 spectrophotometer high speed centrifugations have been done on Louder LRA refrigerated centrifuge

Results

We have assayed in the erythrocytes of the propositus the GSH content and stability glucose uptake and the levels of those enzyme activities showing most frequently a genetic deficiency The results are shown in table I and compared to normal values of newborn and adult subjects. The glucose-6-phosphate dehydrogenase 6-phosphogluconate dehydrogenase and triose phosphate isomerase activities showed normal values the GSH content is slightly decreased, but the stability test is normal as expressed in per cent values. On the contrary glucose uptake (46 the normal values) the synthesis of lactate (48 the normal values) and the pyruvate kinase activity (18 the normal values) are markedly decreased.

These metabolic alterations and laboratory findings indicate therefore that the hemolytic episode of the propositus is due to a PK deficiency The slight decrease of GSH content my be referred to the marked lowering of glucose uptake

We have assayed PK activity also in the young and old erythrocytes which were separated by hypotonic hemolysis. Also in both of these

Table I Erythrocyte metabolic activities of the propositus, and of normal newborns and adults

Metabolic activities		Propositus	Normal subjects Newborn		Adults	
Reduced glutathione	before APH	63	74	± 5.8	72	4 6
	after APH	5_	61	± 7.2	_9	5 7
Glucose uptake		1.8	3.4	± 0.4	2.6	0 3
Lactate synthesis		2.7	5.6	± 0.7	4.5	0 6
G-6-P dehydrogenase		73	72	± 8.3	5[illegible]	± 6 1
6-P-G dehydrogenase		60	63	± 7 4	43	
Pyruvate kinase		26	196	± 23	153	17
Trioso-P-isomerase		4600	4.00	6.0	4300	520

The reduced glutathione content is expressed in mg 100 ml packed erythrocytes. The glucose uptake lactate synthesis and enzyme activities are referred as µmoles of metabolized substrate hour ml packed erythrocytes. The values for normal subjects are the means of literature and personal data.

fractions the enzyme activity was low being 47 units in the young cells and 19 units in the old ones.

The clinical course and the very low level of PK activity of the propositus raised the possibility of an homozygous state of the defect. Therefore we have assayed erythrocyte PK activity also in the relatives of the propositus in those cases where the enzyme activity was decreased, glucose uptake also has been determined. Table II and figure 1 show the results of the investigations performed on the relatives. The defect of PK is present in the erythrocytes of the paternal branch whereas the maternal branch is unaffected. In the affected subjects the defect appears to be in the heterozygous state the PK activity ranged between 44–70% the normal values. Glucose uptake and lactate synthesis were slowly decreased in two subjects only (case No 10 and No 16)

Table II. Erythrocyte pyruvate kinase activity and glucose utilization in the family members of the propositus

Subjects Case No.	Pyruvate kinase	Glucose utilization glucose uptake	lactate synthesis
1	84	2.7	4.8
2	168	–	–
3	153	–	–
4	108	2.5	4.3
4	138	–	–
5	174	–	–
5	142	–	
6	68	2.6	4.6
7	160	–	
8	149	–	–
9	93	2.7	4.3
10	71	2.1	3.4
11	134	–	–
12	172		–
13	165		
15	147	–	
16	87	2.0	3.1
17	159		
18	144	–	–
19	161		

The values are referred as µmoles of metabolized substrate/hour/ml packed erythrocytes. The relationship of the reported cases is elucidated in figure 1

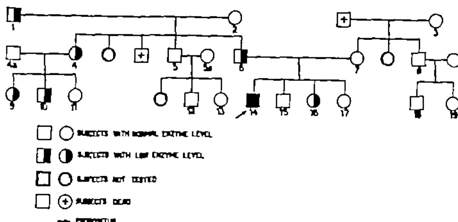


Fig 1 Pedigree of propositus family in which members were tested by the pyruvate kinase ctivity

As it is reported in literature PK deficiency is highly heteroge neous as concerns the activity level [13–16] Since in some hetero-zygous subjects the activity is much lower than in homozygous sub-jects, the propositus could fall within this group However it is also possible that the severity of the defect was related to the newborn state. The results reported in figure 2 are consistent with the latter hypo-thesis.

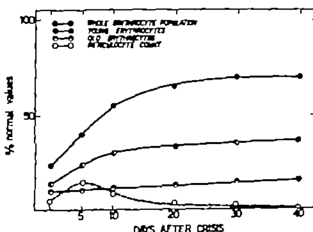


Fig. 2. Pyruvate kinase activity in erythrocyte fractions of different age obtained from the propositus at different time intervals after crisis.

The erythrocyte pyruvate kinase activity of the propositus has been assayed during 40 days at different time intervals on the whole ery throcyte population and on the young and old erythrocyte fractions. The results are reported in the figure 2 as per cent of similar cell preparations obtained from newborns of the same age. In the old erythrocytes the enzyme activity is very low at every time. The enzyme from the WEP in which the reticulocyte count is very high, shows high levels of activity at the 5th and 10th day this increase is also present and progressive in the following period of observation during which blood reaches normal pictures. In the young erythrocytes the enzyme shows marked modifications with aging the activity rises rapidly during the first 20 days and then becomes stable at a level (66% the normal) which is the mean value in the heterozygous subjects.

Discussion

Many authors [17–19] have demonstrated that some transitory modi fications of glucose-6-phosphate dehydrogenase induce neonatal hemolysis. Our present results show that this may be also for pyruvate kinase in the propositus the transitory state of deficiency comes out to be a greater extent of the inherited trait. The low levels of the enzyme activity could account for a homozygous state. A rather long time interval is necessary for observing the disappearance of the transitory state of the defect thus, in the young cells the enzyme level reaches values which are typical for the heterozygous state only after more than 20 days from the hemolytic episode.

Few evidences have been provided that a transitory state of defi ciency affecting both normal or genetically altered enzymes, may de termine hemolysis. Many reasons may account for this. The hemolysis in the neonatal period has been considered only recently from a biochemical point of view In few cases the biochemical features have been investigated during the hemolytic episode also in these cases those parameters have been examined which are usually affected by genetic mutations. In most cases, where transfusions are made, the biochemical assays are performed a long time after the crisis and therefore it is possible to detect only the metabolic inherited defects in their typical expressions.

Many authors [20–27] have incriminated some endogenous factors other than the genetic defect for the hemolytic episode in newborn

with G-6-PD deficiency The hemolysis, which is known to affect approximately 5% of infants bearing this defect [27] has been shown to be independent of the degree of the deficiency moreover no known hemolytic agents could be demonstrated to determine the episode. For these cases the additional, indipendent genetic factor might be a transient state of deficiency

The mechanisms determining the transient states of deficiency are unknown However the occurrence of this condition is probably related to the features of the newborn s erythrocyte which are very different from those of the adult[28] The newborn s red blood cells, besides for the HbF differ from the adult's ones as concerns electron microscopic appearance and chemical composition of the membrane, carbohydrate uptake and the activity levels of several enzymes. On this subject it must be remembered that the marked vulnerableness of newborn s cells to damage from hydrogen peroxide [29 30] and from oxidizing agents [31–35] may be ascribed to the lowered levels of glutathione peroxidase [30] and katalase [36] activities.

As regards the mechanisms of hemolysis due to PK deficiency this may be framed in the general problem of that hemolysis which related to metabolic defects. It has been established that the process leading to sequestration and destruction of erythrocytes in the reticuloendothelial system, is determined by a progressive failure in energy production [37–43] and in the maintenance of the reductive state [44–49] of the cell. Both these processes are related to glucose utilization which is the only fully active metabolic process in the mature circulating cell. Energy production occurs by ATP resynthesis at the pyruvate kinase and phosphoglycerate kinase steps the maintenance of the reductive state is linked with reduced pyridine nucleotides synthesis through the dehydrogenase reactions of the Embden Meyerhof Parnas pathway and of the oxidative shunt. Thus, all the erythrocyte defects leading to modifications of either process may induce the hemolytic episode.

As concerns pyruvate kinase, when the defect is partial, that is, in the heterozygous state, the damage is generally asymptomatic, probably because of two reasons 1 Pyruvate kinase shows high levels of activity [41 42, 50] and may work sufficiently even when it is partially damaged 2 the possible compensatory mechanism for ATP synthesis by the Rapoport Luebering cycle [51 52] In fact the enzymes of this cycle (phosphoglycerate kinase, diphosphoglyceromutase, diphosphoglycerate phosphatase) regulate the glycolytic resynthesis of ATP The transformation of 1,3-diphosphoglycerate into 3-phosphoglycerate may

occur through the phosphoglycerate kinase reaction with coupled ATP resynthesis or through diphosphoglyceromutase and diphosphoglycerate phosphatase reactions with release of inorganic phosphate.

In those cases where a total defect is present the pyruvate kinase activity is so low as to block the glycolytic process, as it is clear from the decreased glucose uptake. This damage leads to a decreased ATP and pyridine nucleotides synthesis and the alterations occur (cell shape, permeability, phospholipid, cholesterol and coenzyme turnover, allosteric properties of Hb, oxidation of Hb and of structural and enzyme proteins) determining the lysis.

Summary

A case of hemolysis in a 4 days old infant is reported which is due to a transient severity of a heterozygous pyruvate kinase deficiency. Assays performed on the whole erythrocyte population during the crisis revealed normal levels of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and triose-phosphate isomerase, a low decrease of GSH content without any modifications of its stability, a marked decrease of pyruvate kinase activity (18% the normal values) and of glucose uptake (50% the normal values). The pyruvate kinase activity has been followed during 40 days from the beginning of the hemolytic episode on the whole erythrocyte population and on young and old cell fractions. It has been demonstrated that in the young cells the enzyme activity increases progressively till reaching a level (66% the normal values) which is peculiar of the heterozygous state of the defect.

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Die Forschungen auf dem Gebiet der Gewebe- und Organtransplantationen machten in den letzten Jahren ausserordentlich grosse Fortschritte. Die neuen Erkenntnisse ergaben sich aus Arbeiten verschiedenster Spezialitäten, wie Immunologie, Biochemie, Genetik, innere Medizin und Chirurgie. Es erwies sich deshalb als notwendig, dass sich die Forscher aller Spezialitäten, die an den Errungenschaften beteiligt sind, regelmässig treffen. Aus diesem Grunde fand vom 27.–30. Juni 1967 in Paris der 1. Internationale Kongress der Transplantations-Gesellschaft statt. Zuerst wurden an sieben Meetings allgemeine Themen behandelt wie: Der Mechanismus der Transplantationsabstossung – Immunosuppressive Methoden – Genetische Probleme der Transplantation – Transplantationsantigene – Organtransplantation – Knochenmarktransplantation – Krebs als Transplantat u. Danach folgten verschiedene Übersichtsreferate, u.a. Betrachtungen zum Abstossungsmechanismus – Spenderauswahl für Organtransplantationen – Biologische Probleme bei Transplantationen – Immunosuppressive Substanzen u. Weitere Referate befassten sich mit Transplantationen von Niere, Leber, Herz, Lunge, Pankreas und Gehirn.

Die im Kongressband niedergelegten Ergebnisse sind nur für den an Transplantationsproblemen speziell interessierten Mediziner bestimmt. Derjenige, der sich über Transplantationen allgemein orientieren möchte, sollte nicht zu diesem Buch greifen. Dem Spezialisten dagegen bietet es eine grosse Fülle von neuen Erkenntnissen.

H. Thölen, *Basel*

Varia

VIIth International Congress of Clinical Pathology

The VIIth International Congress of Clinical Pathology will be held in Montreal, July 13th to 19th, 1969 under the joint auspices of the Canadian Association of Pathologists, the Canadian Society of Clinical Chemists and the Canadian Association of Medical Bacteriologists.

Tentative scientific programme. Monday July 14: Symposium I: Mycoplasma; Symposium II: Enzyme Tests. – Tuesday July 15: Symposium III: Complications of Extra-Corporeal Perfusion and Cardiac Surgery; Symposium IV: Standards, International Surveys and Quality Control; *Symposium V: Immunofluorescence in Diagnosis.* – Thursday July 17: Symposium VI: Restrictive Lung Disease; *Symposium VII:* Hospital Infections; *Symposium VIII:* Neuropathology. – Friday July 18: Symposium IX: Inborn Errors in Metabolism; Symposium X: Cytology; *Symposium XI:* Haemolytic *Disease* of the Foetus and *Newborn.* – *Saturday July 19: Symposium XII:* Future of Laboratory Medicine.

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Pathologisches Institut der Universität Köln (Direktor: Prof. Dr. M. Eder) und II. Medizinische Universitätsklinik und Poliklinik Mainz (Direktor: Prof. Dr. P. Schölmerich)

Über den Nachweis der Thiaminpyrophosphatase («Golgiphosphatase») in Blut- und Knochenmarkszellen[1]

R. Fischer und H. H. Hennekeuser

Novikoff und Goldfischer [17] haben eine enzymcytochemische Methode angegeben, mit der in den Zellen zahlreicher Gewebe im Bereich der Golgizone ein deutlicher Reaktionsausfall feststellbar war. Bei diesem auf dem Gomoriprinzip basierenden Nachweisverfahren dienten verschiedene Nukleosiddiphosphate sowie Thiaminpyrophosphat als Substrate, die bei annähernd neutralem pH von einer «Golgi-Phosphatase» hydrolisiert wurden. Die Annahme einer solchen spezifischen Enzymlokalisation in den lamellären und vesikulären Strukturen des Golgiapparates konnte in der Folgezeit bestätigt werden [2, 5, 13, 15, 16, 20].

Bei den vorliegenden Untersuchungen sollte das Vorkommen und die Lokalisation der Thiaminpyrophosphatase in den Zellen des Blutes und Knochenmarks geprüft werden. Die Klärung dieser Frage erschien vor allem deshalb von Interesse, weil sich gerade in jüngster Zeit wichtige Hinweise für die funktionelle Bedeutung des Golgiapparates bei der Granulabildung in den Zellen des hämatopoetischen Systems ergeben haben [3, 7, 23].

Material und Methodik

Als *Untersuchungsmaterial* dienten Blut- und Knochenmarksausstriche von 30 gesunden Personen bzw. von Patienten mit nichthämatologischen Erkrankungen. Außerdem wurden 20 Fälle verschiedener Leukämien untersucht.

[1] Mit Unterstützung durch die Deutsche Forschungsgemeinschaft. Auszugsweise vorgetragen auf der 12. Tagung der Deutschen Gesellschaft für Hämatologie, Berlin 1966.

Die Ausstriche wurden luftgetrocknet und anschließend fixiert. Unter den in Vorversuchen getesteten *Fixierungsmitteln* ergab die Vorbehandlung mit Formalin-Calciumchlorid oder Formalin-Calciumacetat (5 Min., 4° C) die besten Resultate.

Nach der Fixierung wurden die Ausstriche in Aqua dest. gespült und erneut luftgetrocknet.

Die *Inkubation* erfolgte 1 Std. bei 37° C in dem von Novikoff und Goldfischer [17] angegebenen Medium: 20 ml 0,01 M Thiaminpyrophosphat, 8 ml Aqua dest., 40 ml 0,2 M Tris-Maleat-Puffer pH 7,2, 12 ml 0,03 M Bleinitrat und 20 ml 0,025 M Manganchlorid.

Nach der Inkubation wurden die Präparate mehrmals in Aqua dest. gespült, anschließend für 1–2 Min. in verdünnte Ammoniumsulfidlösung eingestellt und erneut gewässert. Die Kerngegenfärbung erfolgte mit Mayers Hämalaun. Eindecken in Glyceringelatine.

Die Spezifität der Thiaminpyrophosphatase-Aktivität wurde durch folgende *Kontrollreaktionen* geprüft: a) Weglassen des Substrates oder Hitzeinaktivierung, b) Zusatz von folgenden Inhibitoren zum Inkubationsgemisch: 0,01 M Uranylnitrat, 0,01 M Natriumfluorid, 0,01 M Zysteinchlorid. Nach Weglassen des Substrates, Hitzeinaktivierung sowie nach Zusatz von Uranylnitrat war keine Enzymaktivität feststellbar. Dagegen blieb die Aktivität der Thiaminpyrophosphatase durch die Inhibitoren der sauren Phosphatase (Natriumfluorid) und der alkalischen Phosphatase (Zysteinchlorid) unbeeinflußt.

Neben der Pappenheim-Färbung kamen an Ausstrichpräparaten der untersuchten Fälle in parallel durchgeführten Untersuchungen außerdem folgende enzymzytochemischen Nachweisverfahren zur Anwendung: Naphthol-AS-D-Chloracetat-Esterase [14], Naphthol-AS-Acetat-Esterase [12], saure Phosphatase [4], alkalische Phosphatase [11].

Ergebnisse

In der Entwicklungsreihe der *neutrophilen Granulozyten* zeigen Myeloblasten und frühe Promyelozyten nur eine geringe Aktivität der Thiaminpyrophosphatase, deren Reaktionsprodukt hier in einem kleinen umschriebenen, perinukleären Zytoplasmabezirk lokalisiert ist (Abb. 1a). Mit der weiteren Differenzierung zu Promyelozyten fällt *eine deutliche Vergrößerung der Aktivitätszone auf*, die relativ scharf begrenzt in der Nähe der beginnenden Kerneinbuchtung liegt (Abb. 1b und c). Das Reaktionsprodukt zeigt in den Promyelozyten häufig eine ring- bzw. schalenförmige Anordnung, deren Mitte etwa der Lage der Zentriolen entspricht (Abb. 1b). Auch im Myelozytenstadium findet sich zumeist eine deutliche Fermentaktivität, deren Ausdehnung im Vergleich zu den Promyelozyten etwas abgenommen hat (Abb. 1d und e). *Noch deutlicher wird die Abnahme der Enzymaktivität bzw.* die Verkleinerung der Reaktionszone mit der weiteren Ausreifung der neutrophilen Granulozyten. Während in den Metamyelozyten häufig noch einzelne Bleisulfidniederschläge zu beobachten sind (Abb. 1f), war in den stab- und segmentkernigen Neutrophilen in der Regel keine sichere Enzymaktivität mehr nachweisbar.

Die Aktivitätszone der Thiaminpyrophosphatase in den Promyelozyten entspricht der im panoptisch gefärbten Präparat erkennbaren Lokalisation der beginnenden Azurgranula-

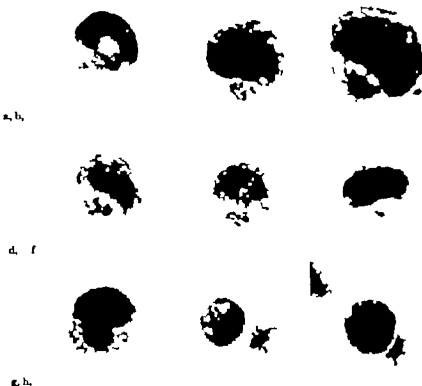


Abb. 1. Nachweis der *Thiaminpyrophosphatase* in Zellen des menschlichen Knochenmarks a) Myeloblast, b) und c) Promyelocyten, d) und e) Myelocyten, f) Metamyelocyt, g) eosinophiler Myelocyt, h) und i) Plasmazellen. Relativ scharf lokalisierte Reaktion in einem perinukleären, der Golgizone entsprechenden Bezirk. × 1400.

tion. Eine ähnliche, zunächst auf einen perinukleären Bezirk beschränkte Reaktion zeigt im frühen Promyelocyten auch der zytochemische Nachweis der *Naphthol-AS-D-Chloracetat-Esterase* (Abb. 2) oder anderer an die Leukocytengranula gebundener Enzyme (Peroxydase, Naphthol-AS-Acetat-Esterase, saure Phosphatase).

Der Reaktionsausfall in den *eosinophilen Granulocyten* stimmt im Prinzip mit den geschilderten Befunden an den Zellen der neutrophilen Entwicklungsreihe überein. Im allgemeinen fällt der Nachweis der Thiaminpyrophosphatase in den Eosinophilen jedoch etwas kräftiger aus als in den entsprechenden neutrophilen Zellen. Besonders deutlich tritt die charakteristische zirkuläre Orientierung der Aktivitätszone in den eosinophilen Promyelocyten und Myelocyten hervor



Abb 2. Darstellung der *Naphthol-AS-D-chloracetat-Esterase* in frühen Promyelozyten mit einem zunächst auf einen perinukleären Bezirk (Golgizone!) beschränkten Reaktionsprodukt (und b) das sich bei der weiteren Differenzierung der Zellen auf größere Abschnitte ausdehnt (c) und schließlich das Zytoplasma völlig ausfüllt (d). Promyelozyten-Leukämie. × 1400.

(Abb 1g) Außerdem war im Gegensatz zu den enzymnegativen stab- und segmentkernigen Neutrophilen auch in den reifen eosinophilen Leukozyten gelegentlich noch eine geringe Aktivität der Thiaminpyrophosphatase in Form einzelner punktförmiger Granula zwischen den beiden Kernsegmenten nachweisbar

Durch eine besonders starke Thiaminpyrophosphatase Aktivität fallen die in den Knochenmarkausstrichen nachweisbaren *Megakaryozyten* auf. Das Reaktionsprodukt ist in den Vorstufen mit noch unsegmentiertem Zellkern in einer deutlich ausgeprägten perinukleären Zone lokalisiert (Abb 3a) Besonders bei stärkerer Vergrößerung ist hier eine lamelläre und granuläre Anordnung feststellbar Mit zunehmender Kernlappung und Ausdifferenzierung der Megakaryozyten dehnt sich die Aktivitätszone über größere Anteile des Zytoplasmas aus (Abb 3b und c) Neben unregelmäßigen Verdichtungen des Reaktionsproduktes lassen sich netzförmige Strukturen oder granuläre bzw feinvakuoläre Reaktionsniederschläge in kettenförmiger Anordnung feststellen.

Sowohl in den Zellen der *Erythrozytopoese* als auch in den *Lymphozyten* im peripheren Blut und Knochenmark war mit der von uns ange



Abb. 3. Deutliche Aktivität der *Thiaminpyrophosphatase* in Megakaryocyten. Das Reaktionsprodukt ist in einer perinukleären Zone ( ) mit zunehmender Kernlappung in einem größeren Zytoplasmabezirk (b) z.T. in netzförmiger bzw. feinvakuolärer Anordnung ( ) erkennbar. × 900

wandten Technik kein sicherer Reaktionsausfall der Thiamin-pyrophosphatase nachweisbar. Eine zumeist deutliche Enzymaktivität

n einer umschriebenen etwa der perinukleären Aufhellung entsprechenden Zone fand sich dagegen in den *Plasmazellen* des Knochenmarks Abb 1h und i) *Monozyten* des peripheren Blutes zeigten eine insgesamt schwache Reaktion im Bereich der Kerneinbuchtung

Bei den untersuchten *Leukosen* entsprach die Aktivitätsverteilung der Thiaminpyrophosphatase weitgehend dem Differenzierungsgrad der Zellen So zeigten unreifzellige myeloische Leukämien je nach ihrer Differenzierungstendenz zu promyelozytären Zellen eine unterschiedlich entwickelte Golgiphosphataseaktivität. Auch in den neutrophilen und eosinophilen Promyelozyten und Myelozyten der chronischen Myelose bestand, ähnlich wie in den entsprechenden Zellen des normalen Knochenmarks, eine deutliche Enzymaktivität. Die Zellen lymphatischer Leukämien wiesen keine zytochemisch nachweisbare Aktivität der Thiaminpyrophosphatase auf.

Diskussion

In der seit langem geführten Diskussion über die Existenz und die funktionelle Bedeutung des in enger struktureller und funktioneller Beziehung mit dem endoplasmatischen Retikulum stehenden Golgiapparates konnte inzwischen eine weitgehende Klärung herbeigeführt werden [Übersichten 9 und 21] Vor allem die neueren Ergebnisse der Elektronenmikroskopie haben gezeigt, dass Strukturen des Golgisystems, allerdings in sehr unterschiedlicher Ausprägung eine obligatorische Komponente der tierischen Zellen darstellen. Zur lichtmikroskopischen Darstellung des Golgiapparates hat sich in jüngster Zeit besonders der von NOVIKOFF und GOLDFISCHER [17] eingeführte zytochemische Nachweis einer Nukleosiddiphosphate bzw Thiaminpyrophosphat hydrolysierenden Phosphatase bewährt, deren spezifische Lokalisation in Strukturen des Golgisystems inzwischen durch zahlreiche licht und elektronenmikroskopische Untersuchungen an verschiedenen Geweben bestätigt worden ist [2, 5, 13 15 16, 20]

Die vorliegenden Ergebnisse zeigen, dass die zytochemische Lokalisation der Thiaminpyrophosphatase auch in den Zellen des hämatopoetischen Systems mit der aus elektronenmikroskopischen Untersuchungen bekannten Anordnung des Golgiapparates völlig übereinstimmt In der neutrophilen Granulozytopoese findet sich das Maximum der Enzymaktivität im Promyelozyten- und Myelozytenstadium,

während in den reifen Neutrophilen mit der angewandten Methode keine nennenswerte Reaktion mehr nachweisbar war. Auch elektronenmikroskopisch zeigt der in Myeloblasten nur spärlich ausgebildete Golgiapparat in den Promyelozyten und Myelozyten eine deutliche Vergrösserung, um sich mit der weiteren Ausreifung bis auf einige rudimentäre Zysternen wieder zurückzubilden [1, 3, 6, 22]. Entsprechende Übereinstimmungen ergeben sich auch in der eosinophilen Entwicklungsreihe.

Der besonderen Ausprägung des Golgiapparates im Promyelozyten- und Myelozytenstadium muss in Übereinstimmung mit den allgemeinen Vorstellungen über die funktionelle Bedeutung dieses Zellorganells eine wichtige Rolle bei der Granulabildung zugesprochen werden, die in enger räumlicher Beziehung zu Strukturen des Golgikomplexes steht [1, 3, 7, 23]. So entwickeln sich beispielsweise in den neutrophilen Vorstufen im Knochenmark des Kaninchens die azurophilen Granula durch Ausstülpung und nachfolgende Aggregation von Mikrobläschen an der konkaven, d. h. der dem Zentriol zugewandten Fläche des Golgiapparates, während die im Myelozytenstadium auftretenden «spezifischen» Granula durch Abschnürung und Konfluenz von Golgibläschen an der konvexen Begrenzung entstehen [3]. In Übereinstimmung mit diesen elektronenmikroskopischen Befunden stehen die Ergebnisse autoradiographischer Untersuchungen, bei denen nach Inkorporation von H Lysin der intrazelluläre Fluss der markierten Aminosäure von Strukturen des endoplasmatischen Retikulums über den Golgikomplex in die neugebildeten Granula verfolgt werden konnte [7].

Tatsächlich lässt sich auch lichtmikroskopisch in frühen Promyelozyten im Bereich der zytochemisch darstellbaren Golgiphosphatase nicht nur im panoptisch gefärbten Präparat der Beginn einer azurophilen Granulation nachweisen, sondern auch eine zunächst auf diese Zone beschränkte Aktivität bestimmter an die Leukozytengranula gebundener Enzyme wie der Peroxydase oder der Naphthol-AS-D-Chloroazetat Esterase (Abb. 2) feststellen. Eine ähnliche Lokalisation der sauren Phosphatase und anderer hydrolytischer Enzyme [1, 23] liefert zudem einen weiteren Beleg für die Lysosomennatur der Leukozytengranula sowie Hinweise für die Bedeutung des Golgikomplexes bei der Entstehung «primärer» Lysosomen überhaupt. Dabei ist die saure Phosphatase jedoch im Gegensatz zu der Nukleosiddiphosphate oder Thiaminpyrophosphat hydrolisierenden «Golgiphosphatase» nicht in den lamellären Strukturen des Golgiapparates selbst lokalisiert

sondern in den benachbarten lysosomalen Granula anzutreffen [16 17].

Eine besonders auffällige Aktivität und Verteilung der Thiaminpyrophosphatase liess sich bei unseren Untersuchungen in den Megakaryozyten des Knochenmarks feststellen. Sie entspricht auch hier den elektronenmikroskopischen Befunden eines sehr stark ausgeprägten Golgiapparates, der bereits in den Megakaryoblasten im perinukleären Zytoplasma eine grosse, aus mehreren herdförmigen glatten Membranen und vielen Mikrobläschen aufgebaute Zone bildet. Unter wesentlicher Beteiligung des Golgiapparates entstehen in den Megakaryozyten aus Eiweissbausteinen der Grundsubstanz die verschiedenen Vorstufen des Granulomers und der Demarkationsbläschen der Thrombozyten [10 18, 19].

Auch in den Zellen des hämatopoetischen Systems kommt somit dem Golgiapparat eine wesentliche Bedeutung im Rahmen «sekretorischer» Leistungen zu wie sie etwa von den Plasmazellen oder Geweben mit einer starken Eiweissynthese bereits seit längerem bekannt ist. Durch die Thiaminpyrophosphatase Reaktion ergibt sich nicht nur die Möglichkeit, die Struktur des Golgiapparates in den Zellen des Blutes und Knochenmarks mit einer den klassischen Imprägnationsverfahren überlegenen enzymzytochemischen Methode darzustellen sondern auch seine Variabilität unter funktionellen Bedingungen, wie etwa bei den Transformationsvorgängen der Blutlymphozyten *in vitro* [8] zu erfassen. Vor allem durch die Kombination mit anderen Enzymnachweisen werden darüber hinaus auch auf zytochemischer Basis weitere Hinweise auf die Bedeutung des Golgikomplexes bei der zellulären Differenzierung der blutbildenden Systeme möglich sein.

Zusammenfassung

Der zytochemische Nachweis der Thiaminpyrophosphatase in Zellen des Knochenmarks zeigt eine unterschiedlich starke Enzymaktivität, deren Lokalisation mit der aus elektronenmikroskopischen Untersuchungen bekannten Anordnung und Grösse des Golgiapparates übereinstimmt. In der neutrophilen und eosinophilen Entwicklungsreihe zeigen Promyelozyten und Myelozyten das Maximum der Enzymaktivität. Durch eine besonders ausgeprägte Reaktion der Golgiphosphatase sind die Megakaryozyten gekennzeichnet. Ausserdem ist eine zumeist deutliche Enzymaktivität in den Plasmazellen feststellbar. Der Thiaminpyrophosphatase und der besonderen Ausprägung des Golgiapparates in bestimmten Knochenmarkzellen muss eine wichtige Rolle bei den «sekretorischen» Leistungen dieser Zellen, vor allem bei der Granulabildung, zugesprochen werden.

Summary

Cytochemical demonstration of thiamine pyrophosphatase in bone marrow cells reveals different degrees of enzyme activity according to the arrangement and size of the Golgi apparatus known from electron microscope studies. Among the neutrophils and eosinophils, maximum activity is found in the promyelocytes and myelocytes. Megakaryocytes are characterized by particularly marked Golgi phosphatase reaction. Fairly marked activity is also generally seen in the plasma cells. The presence of thiamine phosphatase and the particular development of the Golgi apparatus in certain bone marrow cells must play an important part in the secretory activity of these cells, especially formation of the granula.

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Serum Lactic Dehydrogenase Activity in Refractory Anemia[1]

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Serum lactic dehydrogenase activity (LDH) is markedly elevated in patients with untreated megaloblastic anemias secondary to folic acid or Vitamin B_{12} deficiency and this activity rapidly falls with appropriate therapy [1–4]. The serum LDH levels in the anemias of iron deficiency and bone marrow aplasia are normal but levels in hemolytic anemias are frequently elevated [3]. Over the past several years we have been following a group of patients with refractory anemias, some with ineffective erythropoiesis and demonstrating sideroblasts, others presenting with Di Guglielmo's syndrome. It is often difficult to distinguish morphologically the megaloblastoid changes in these cases from that noted in pernicious anemia or folate (FA) deficiency. In this paper results of studies on the behavior of serum LDH and LDH isozymes will be reported.

Methods and Materials

There were 9 patients in this series, all characterized by severe anemia, low reticulocytes, variable red cell morphology in the peripheral blood and bone marrow showing marked erythroid activity with reversal of the myeloid to erythroid ratio. Red cells were of varying degrees of immaturity ranging from pronormoblasts with normal maturation to a population of primitive erythroid precursors associated with multi-nucleated dysplastic normoblasts. Megaloblastic like changes were frequently noted. In 7 of the 9 cases Prussian Blue stain demonstrated ringed sideroblasts and generally increased iron deposition in phagocytic histiocytes. In some cases there was an increased number of promyelocytes and myelocytes, but a striking absence of macrometamyelocytes and other myeloid changes usually associa-

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ted with B_{12} or folate deficiency Over several months, 3 patients progressed to overt acute granulocytic leukemia and expired. Six patients were refractory to therapy two of which required frequent transfusions (table I). Serum LDH activity was measured by the methods of Wroblewski [5] and the percentage of serum LDH isoenzyme ('Heart' type) by the method of Dawson *et al.* [6].

Results

There were no significant differences in the LDH levels or the percent of LDH isozymes in the serum of a group of 15 normal controls compared to the patients with refractory anemia (fig 1) The mean serum LDH level for normals was 221 5 units and for the patients 241 units.

Discussion

The extremely high serum LDH level reported in megaloblastic anemias associated with B_{12} and FA deficiency was not present in patients with refractory anemia characterized by increased iron and ringed sideroblasts. These findings are in agreement with observations made by Hoffbrand *et al.* [7] The elevated serum LDH activity in megaloblastosis has been presumed to come from either increased hemolysis or directly from cell breakdown in the bone marrow [2, 3]

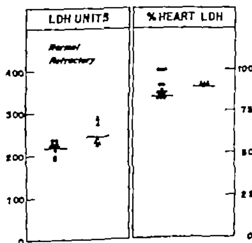


Fig 1 Serum LDH and LDH isoenzyme activity in 9 patients with refractory anemia and 15 normal controls.

Table I Clinical and laboratory data in nine cases of refractory anemia

Case No	Age/Sex	L/D	1st seen	Death	Hb g%	Hct %	WBC	Retic %	Bone marrow	Folic acid ng	B_{12} pg	Fe µg	LDH w.u	H %
1	67 ♂	+	11/30/64		7.5	24	2850	1.4	Ringed siderobl.	300	849	197	277	91
2	72 ♀	+	12/23/63		9.8	25	1800	2.2	Ringed siderobl.	5.5	689	120	312	91
3	74 ♂	+	6/28/66		8.2	25	3000	2.2	Ringed siderobl.	225	671	224	222	68
4	80 ♂	—	7/1/66	10/13/68	8.5	27	7200	1.8	Ringed siderobl.	30	1823	–	340	–
5	56 ♀	+	10/31/68		7.5	25	5500	2.5	Ringed siderobl.	17.2	681	249	228	84
6	67 ♂	+	11/30/66		8.8	26	4000	0.4	Ringed siderobl.	3.6	383	100	142	91
7	42 ♀	—	12/28/66	5/31/67	10.0	32	1450	3.0	Incr F	–	–	171	225	75
8	43 ♀	—	1/10/67	5/19/67	6.0	19	2600	1.8	Incr F	11.9	745	117	241	100
9	48 ♂	+	9/13/67		9.6	28	7600	3.2	Ringed siderobl.	40	2080	172	290	–
									Normal values	7–15.9	200–900	50–150	221.5	84

+ living, — dead
Converted to acute granulocytic leukemia
Wroblewski units

This increase has been attributed to an absolute increase in LDH_1 and LDH_2 the two elektrophoretically fast LDH isoenzymes [3] found in adult red cells. In the refractory anemias there are probably two defects an 'ineffective' type of erythropoiesis of marked degree and hemolysis resulting from increased destruction of defective red cells in the bone marrow [8]. The normal serum LDH levels in these 9 patients is of diagnostic importance and raised the question as to the source of elevated LDH activity in megaloblastic anemias.

Our observations suggest that the ineffective erythropoiesis in cases of refractory anemia may not be due to abnormal cellular proliferation, but rather to an accumulation of immature red cells with decreased destruction within the bone marrow. On the other hand, the ineffective erythropoiesis in megaloblastosis may be a result of rapid destruction of immature red cells with release of LDH from the marrow. Recent evidence of ineffective granulocytopoiesis raises the alternative possibility that much of the elevated serum LDH activity may be derived from the myeloid elements in the megloblastic bone marrow [9, 10].

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Summary

Normal serum levels of LDH and its isoenzymes were found in 9 cases of refractory anemia. The red cell activity and morphology in these cases was often indistinguishable from that seen in pernicious anemia. The source of elevated LDH activity in megaloblastosis due to B_{12} and folic acid deficiency may therefore not be derived from the increased erythroid activity but rather the increased myeloid elements which are not present in refractory anemia.

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Cytogenetic Studies in Acute Leukemia[1]

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The discovery of a specific chromosomal abnormality in chronic myeloid leukemia [1, 7, 8] has led to a revival of interest in the karyotyping of human tumors. In this respect the acute leukemias have received special attention, at least partly because of the difficulties frequently encountered in classifying them on cytological grounds alone.

The present report deals with the results of cytogenetic studies on bone marrow aspirates from 7 consecutive patients admitted to the American University Hospital with the diagnosis of acute leukemia.

Materials and Methods

Six patients were males and one was a female. The age range was 16–63 years, with only one patient (LCP 3) above the age of 45 years. Four had received no treatment prior to karyotyping: one (LCP 14) had prednisolone for one month, another (LCP 18) had nitrogen mustard for 7 days, 2 months prior to the examination, and a third patient (LCP-7), the only female in the group, was on both prednisolone and 6-mercaptopurine at the time of the investigation.

As to the cytological type, 3 of the patients had myeloblastic, three lymphoblastic and one monoblastic leukemia. The criteria used in differentiating these types have been described in detail by Hayhoe *et al.* [3]. Features of diagnostic importance were cellular and nuclear morphology, nuclear-cytoplasmic ratio, the presence of certain cytoplasmic inclusions such as Auer rods, as well as cytochemical reactions of the leukemic cells.

Sternal bone marrow aspiration was performed on every patient at the time of admission. A small portion of the aspirate was used for cytological examination following coloration with the Romanowsky stains, and for cytochemical reactions [3]. The rest was utilized in the

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cytogenetic studies using a slight modification of the method of Tjio and Whang [11], without prior in vitro culturing (direct marrow preparations). At the same time peripheral leucocyte counts were made and smears prepared for differential counts and for cytochemical studies.

Table I contains the pertinent clinical and hematological data.

Results

The results of the chromosome studies are shown in table II. In 4 of the patients all available plates were suitable for analysis, and all showed an entirely normal chromosome constitution. In the fifth patient (LCP 18) most of the plates were found to be normal; only 3 were hypodiploid, with a random loss of one or more chromosomes.

Distinct abnormalities were present in the marrow aspirates from 2 patients. The first, LCP 3, an 83-year-old male with myeloblastic leukemia, had not received any treatment prior to the study. Smears from his bone marrow showed numerous pleomorphic blasts with frequent mitoses, and several macroblasts and binucleate cells. Very few megakaryocytes could be seen. Cytogenetic studies revealed the presence of 2 cell-lines: a major line with a chromosome count of 41 (fig. 1) and a minor near-tetraploid line of cells with 82 chromosomes. None of these latter were of adequate quality for accurate analysis.

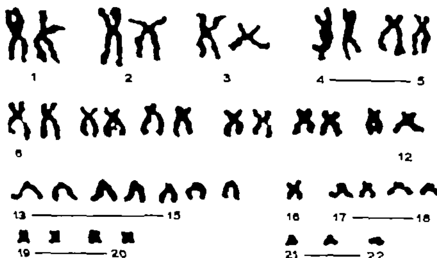


Fig. 1. Case LCP-3. Chromosome constitution of a hypodiploid bone marrow cell. Note missing Y chromosome, extra D and deficiencies in groups C, E and G.

Table I Clinical and hematological data

Patient	Age years	Sex	Duration of symptoms prior to admission	Treatment prior t karyotyping	Leucocyte count	Peripheral blood	Sternal arrow
LCP 3 Case 218156	83	M	3 weeks	None	22,000	Myeloblasts: 73% Promyelocytes: 3% Myelocytes: 4% Metamyelo.: 4%	Numerous pleomorphic blast cells. Mitoses frequent A number of macroblasts' and binucleate blast-like cells. Erythropoiesis markedly depressed. Very few megakaryocytes.
LCP-6 Case 221809	19	M	4 weeks	None	104 000	Lymphoblasts: 82% Lymphocytes: 9%	Numerous lymphoblasts and immature lymphocytes. Granulopoiesis and erythropoiesis depressed.
LCP 7 Case 209810	30	F	8 months	6-mercaptopurine prednisolone	8,550	Myeloblasts: 81% Promyelocytes: 1% Myelocytes: 1%	Numerous pleomorphic blasts, some having Auer bodies. Mitoses freque t. Erythropoiesis relatively active normoblastic. Lymphocytes common

Table I (continued)

Patient	Age years	Sex	Duration of symptoms prior to admission	Treatment prior t karyotyping	Leucocyte count	Peripheral blood	Sternal arrow
LCP-8 Case 222744	43	M	6 months	Vitamins Penicillin-Streptomycin	31,000	Myeloblasts: 80% Promyelocytes: 1% Myelocytes 1%	Numerous blast cells, many having azurophilic granules. Mitoses occasional. Erythropoiesis markedly depressed
LCP 13 Case 223939	16	M	1 week	None	210,000	All cells classified as lymphoblasts	Numerous lymphoblasts and immature lymphocytes. Granulopoiesis and erythropoiesis markedly depressed.
LCP 14 Case 226010	26	M	3 months	Prednisolone 1 month	2,300	Monoblasts and Promonocytes: 81% Neutrophils: 18%	Numerous monoblasts, promonocytes and monocytes. Granulopoiesis markedly depressed. Erythropoiesis relatively active
LCP 18 Case 229125	23	M	3 months	Nitrogen mustard 7 days, 2 months earlier	1,950	Lymphoblasts: 20% Lymphocytes 25%	Numerous lymphoblasts. Mitoses few Granulopoiesis relatively active. Erythropoiesis depressed.

Table II. Results of chromosome studies on sternal marrow aspirates

Patient	Diagnosis	Total cells analysed	40	41	44	45	46	polyploid	Marker chromosome	Remarks
			Chromosome count							
LCP 3	AML	17		13				4 (near tetraploid)	–	Two boormal cell lines
LCP-6	ALL	12					12		–	Normal karyotype
LCP 7	AML	17				7	10		? No. 11	Two abnormal cell lines
LCP-8	AML	14					14		–	Normal karyotype
LCP 13	ALL	5					5		–	Normal karyotype
LCP 14	A. monocytic L.	10					10		–	Normal karyotype
LCP 16	ALL	12	1		1	1	9[1]		–	

Normal karyotype. Missing: 1C. Missing 1 No.2 1 No.17 Missing: 1C, 1D 1 No.16, 2F Y

ALL Acut lymphoid leukemia, *AML* Acut myeloid leukemia.

The second patient, LCP 7 was a 30-year-old female with myeloblastic leukemia. Marrow smears showed numerous pleomorphic blasts, some with Auer bodies. Mitoses were frequent among them. Cytogenetic studies showed two abnormal cell-lines, one with 46 chromosomes and the second with a count of 45 Examples of both lines are shown in figures 2 and 3

Discussion

To date, several hundred cases of acute leukemia have been studied cytogenetically In some the studies were performed on direct bone marrow preparations in others peripheral blood leukocyte cultures were used alone or in combination with the bone marrow preparations. The advantages of direct marrow methods over leucocyte cultures have been stressed by HUNGERFORD *et al.* [4] and more recently by KROGH JENSEN [6] Although marrow specimens in the absence of *in vitro* culturing often yielded fewer mitoses for analysis, the culturing of peripheral leucocytes has frequently resulted in the loss of abnormal cell lines, or in the preferential growth of some cell lines over others. Or if phytohemagglutinin was used in the culture, this stimulated normal lymphocytes to proliferate and predominate the cultured cell population.

Even where no culture methods were used, a variable number of patients with acute leukemia were found to have no demonstrable chromosomal aberrations. In the remaining cases, various abnormalities have been encountered. The overall incidence of these as reported in the world literature was approximately 60% [2] although REISMAN *et al.* [9] were able to demonstrate abnormal cell lines in all specimens studied during the active phase of the disease in children. In our present series only 2 of the 7 patients had karyotype changes in their marrow cells.

The chromosome groups most frequently affected were groups C and G and less commonly groups D E and F Both of our cases had deficiencies in group G and an extra member in group D In addition, case LCP 3 had deficiencies in groups C and E, and one of the lines of case LCP 7 had a missing E.

In contrast with the specific abnormality present in chronic myeloid leukemia, no characteristic chromosome pattern has been established for any type of acute leukemia. In general, it has been found that in lymphoblastic leukemia the abnormal cell lines are usually hyper



Fig. 2. Case LCP-7 Chromosome constitution of the cell line with 46 chromosomes. Note abnormally short? No. 11 deficiency in group G, and extra D.

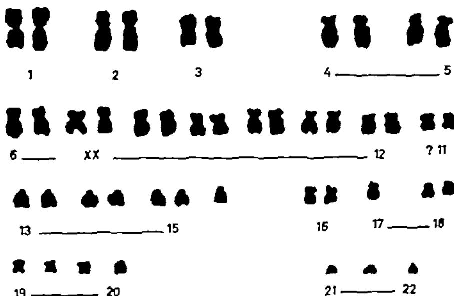


Fig. 3. Case LCP 7 Chromosome constitution of hypodiploid line. Pattern as in figure 2, but in addition, member of pair No. 17 is missing.

diploid, while in the acute myeloid type the abnormal cells are often hypodiploid. An exception to this is the small group of elderly patients with acute myeloid leukemia in whom SANDBERG *et al.* [10] found 2 abnormal lines one hypodiploid and the other hyperdiploid. Our case LCP 3 appears to have many features in common with this group

In conclusion, despite the small size of the series studied our findings are in agreement with the reports in the literature, namely that cytogenetic abnormalities are not demonstrable in many cases of acute leukemia, and if present, they are not of specific diagnostic significance.

Summary

Cytogenetic studies are reported on direct bone marrow preparations from 7 patients with acute leukemia. Abnormalities were present in 2 patients with myeloblastic leukemia, one of whom was receiving cytotoxic drug therapy at the time of the examination. The remaining patients, including 3 with lymphoblastic leukemia, had no demonstrable chromosome changes. The above findings are compared with results of similar investigations carried out elsewhere.

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A Trial of Slow Release Ferrous Sulphate (Ferrogradumet®) in Prevention of Iron Deficiency in Pregnancy

R. W. Burslem, L. Poller and H. Wachs

We have attempted to assess the effectiveness of a slow release ferrous sulphate tablet given once daily as opposed to conventional ferrous sulphate given three times daily in the prevention of anaemia.

An ideal situation for the study of such preparations arises during pregnancy which results in iron deficiency in a considerable proportion of patients when prophylactic iron is not administered. A controlled trial has, therefore, been performed of slow-release ferrous sulphate with folic acid supplement, studied in parallel with a conventional ferrous sulphate tablet and folic acid. A large number of ante-natal patients attending a general maternity hospital has been followed.

Method

The effectiveness of the slow release iron in the prevention of anaemia was evaluated by means of two large parallel groups of ante-natal patients. It was found advisable to use alternate clinics for the selection of patients for the two groups. Two booking clinics every week are held at this hospital. They were identical in all respects.

The series covered initially all patients attending the booking clinic for the first time over a three-month period (September 1965–December 1965). Details were recorded of age, parity, gravidity and previous illnesses. Initial tests included haemoglobin, examination of blood film and urine bacteriology.

Routinely haemoglobin readings are expressed grams per cent. For convenience in the analysis percentage haemoglobin figures were used 100% = 14.6 g with the conventional cyanmethaemoglobin standard.

All patients were given 3 months supply of slow release ferrous sulphate (Ferrogradumet) and folic acid, or combined conventional ferrous sulphate/folic acid. The former group took one tablet daily containing 105 mg elemental iron and one 5 mg folic acid tablet, and the latter group took one tablet three times daily giving a total of 180 mg elemental iron daily and an equivalent dose of folic acid.

Normal patients were not seen again until 30 weeks in the ante-natal clinic following general practitioner care.

From the 30th week of gestation onwards each patient had regular haemoglobin check (capillary finger prick cyanmethaemoglobin) at least once every 4 weeks, although in some cases the haemoglobin was repeated weekly or fortnightly.

If the haemoglobin was below 10.2 g (70%) venous specimen was taken for absolute values. If the venous haemoblogin was found to be less than 10.2 g routine serum iron, B_{12} and folate were advised. I some cases marrow aspiration was performed.

All patients had haemoglobin estimation just prior to delivery and, in nearly all cases within 48 h *post partum*. During the ante-natal period, not was taken of urinary infection, toxaemia, bleeding, hyperemesis and various complications as well as the number of abortions, stillbirths, live births, premature and multiple births and perinatal deaths.

Finally the need for parenteral iron *pre* or *post partum* or any change in the regime of slow release ferrous sulphate to any other form of iron was noted.

Subjective data were not included, e.g. whether the patients said they were taking their tablets, whether they suffered nausea or constipation. It was assumed that with such large numbers this would be reflected in the haemoglobin level.

Results

Of 472 patients in the trial, 228 received slow release ferrous sulphate and folic acid and 224 were given folic acid and ferrous sulphate in conventional combined form. The mean parity of the slow release group was 1.5 and 1 6 with the conventional group The mean age was 26 years in both groups.

Ten patients were lost from each group because of confinement at another hospital, abortions, etc. The percentage of patients given parenteral iron was identical at (11 4%) in both groups. Nine addition al patients were lost from the slow release iron group because they were changed to other oral iron preparations at some stage during the trial.

The effect on haemoglobin levels was as follows. The mean haemoglobin figure after delivery was 79.3% for the slow release groups and 78.7% for the other group. The weekly mean haemoglobin levels are seen in table I Patients who were anaemic had more frequent estima tions of their haemoglobin their results were analysed separately (table II) There were 54 patients with initial haemoglobin of less than 10.2 g in the slow release ferrous sulphate series and 60 patients in the conventional series. Their mean haemoglobins at delivery were 81 1% and 80.3% respectively There was no significant difference in the speed of response of anaemic patients to the two preparations when analysed on a weekly basis using students 't test' (table II) The gross incidence of anaemia in the two groups seemed to be very close and there was no significant difference between the groups.

Table I. Mean Hb levels, %

Weeks	14	28	29	30	31	32	33	34	35	36	37	38	39	40	*Post partum*
Slow release ferrous sulphate	79.4	76.3	78.9	78.0	77.9	78.5	78.12	78.7	80.2	80.2	80.4	81.7	81.7	81.2	79.3
	Mean of 37th–40th week levels = 81.5%														
Ferrous sulphate folic acid	82.6	76.6	79.9	77.9	78.2	77.9	79.3	79.3	81.5	80.4	80.1	80.5	81.5	80.5	78.7
	Mean of 37th–40th week levels = 80.6%														

Table II. Mean Hb levels (%) of patients with 10.2 g and under at initial reading (excluding patients given parenteral iron)

Weeks	Initial	28	29	30	31	32	33	34	35	36	37	38	39	40	*Post partum*
Slow release ferrous sulphate	69.5	70.9	76.5	77.6	76.1	74.9	75.3	78.2	77.6	78.9	79.6	79.7	80.5	79.1	80.6
Ferrous sulphate folic acid	68.2	71.0	72.5	72.4	72.2	72.7	74.7	73.0	75.9	77.3	76.3	78.4	78.9	76.3	79.6

The incidence of complications of pregnancy was as follows. In the slow release iron group urinary infections 19 (8.3%) antepartum haemorrhage 2, hyperemesis 1 in the conventional iron group urinary infections 17 (6.9%) antepartum haemorrhage 4 hyperemesis 1 Premature births were 7 and 6 respectively and the number of ante-natal admissions for various causes was 38 in the slow release iron, and 45 in the other group

Discussion

At the start of the trial the two groups of patients appeared identical. Their ages and parity were similar During the trial obstetrical complications, were similar There was no increase in the number of premature births, abortions or stillbirths. Ante-natal admission rates were similar in the two groups and the number of patients who had hospital admission for investigation of anaemia was insignificant.

The incidence of non-responsive anaemia in patients treated with prophylactic iron and folic acid is of interest. In both series this was 11 4% There is thus a hard core of cases in whom anaemia was not prevented by a prophylactic regime. Similar figures have been reported in the literature. HENDERSON [2] found that regardless of the combination of oral agent used approximately 10% of patients remained anaemic. CHISHOLM [1] in a survey in Oxford found an incidence of anaemic of 12% in spite of prophylactic iron therapy

It appears that the one tablet daily regime of the administration of slow release iron is as effective clinically as giving iron three times daily in conventional form. As the amount of elemental iron is less in the slow release tablets it must be assumed that absorption is more efficient. It does not appear however that this regime is significantly better clinically in the prevention of anaemia.

If the patients acceptability of the new preparation had been superior to conventional iron it might be assumed that the mean haemoglobin would have been maintained at a higher level or that the incidence of anaemia would have been less due to the elimination of cases unable to tolerate conventional ferrous sulphate.

The analysis of the anaemic cases showed that the two preparations were equivalent in their long term effects, no significant difference being found in the two groups at term. Although the differences were not significant the results do perhaps suggest that the response to slow release ferrous sulphate may have been more rapid and this is also

suggested by the higher number of anaemic patients in the conventional ferrous sulphate group who required parenteral iron. Similar accelerated response has been suggested by Israëls and Cook [3] in the treatment of established anaemia. This trial however was not designed to assess the speed of response of established anaemias.

Conventional ferrous sulphate is used on a large scale in hospitals in this country and is consequently extremely cheap. The cost of a combined conventional ferrous sulphate/folic acid tablet for a 28 week course per patient is 4/6d. The preparations of slow release ferrous sulphate (Ferrogradumet®) used in this study is much more expensive, the current cost for a 28 week course at the best hospital price being over 30/ per patient. There might be a case for routine use of the slow release ferrous sulphate if substantial benefit could be shown. In the present study we have found no significant benefit in the prevention of anaemia in pregnancy as distinct from the treatment of established anaemias.

Acknowledgment

We are grateful to Abbott Laboratories for some statistical assistance with the trial and for supplies of Ferrogradumet® and Dr A. Armory Resident Clinical Pathologist, for help in the early stages of the study

Summary

A trial of the effectiveness of slow release, once daily preparation of ferrous sulphate has been performed in the prevention of anaemia in pregnancy The control group consisted of parallel large series of pregnant women given conventional ferrous sulphate three times daily There was no significant difference in the incidence of anaemia, side-effects and complications in the two groups. The once daily regime was as good as, but no better than conventional ferrous sulphate in the prevention of anaemia, although there may be more rapid response in patients with established anaemia.

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Untersuchungen über den Einfluss von Natrium-Oleat auf die Aggregation menschlicher Thrombozyten

A. M. Ehrly, H. Döppenschmidt und E. Böhle

Die Bedeutung der Blutfette für die Entstehung von Thrombozytenaggregaten im Zusammenhang mit Störungen der Hämostase und der Mikrozirkulation ist in den letzten Jahren zunehmend diskutiert worden. Besondere Beachtung verdienen hier die unveresterten Fettsäuren des Plasmas (UFS), die sowohl unter physiologischen als auch unter pathophysiologischen Bedingungen erheblichen Konzentrationsänderungen unterliegen. Über den Einfluss erhöhter Konzentrationen von langkettigen unveresterten Fettsäuren, speziell von Ölsäure auf die Plättchenaggregation liegen allerdings widersprüchliche Angaben vor [1 5 15 16]. Nach eigenen Beobachtungen bewirken sowohl der Zusatz von Ölsäure oder Natrium-Oleat zu Blut *in vitro* als auch eine Erhöhung der Konzentration der unveresterten Fettsäuren *in vivo* eine Desaggregation von Erythrozytenaggregaten [9 10 13]. In der vorliegenden Arbeit wurde untersucht, ob der Zusatz von Natrium-Oleat zu plättchenreichem Plasma eine Aggregation der Thrombozyten *in vitro* bewirkt, und ob verschiedene Konzentrationen von Natrium-Oleat eine ADP induzierte Thrombozytenaggregation verstärken oder abschwächen können.

Material und Methode

Nüchternblut gesunder Probanden beiderlei Geschlechtes zwischen 17 und 41 Jahren wurde durch Punktion der Vena cubitalis gewonnen, wobei die ersten ml verworfen wurden.

Herrn Prof. Dr. W. Siede zum 60. Geburtstag

Das Blut wurde in silikonierten Gläschen aufgefangen, in denen so viel Natrium-Zitrat Lösung vorlag, dass eine Endkonzentration von 3,8% im Blut erreicht wurde. Plättchenreiches Plasma wurde durch 20 min dauerndes Zentrifugieren des Blutes bei 300 g, plättchenfreies Plasma durch 20minütiges Zentrifugieren des plättchenreichen Plasmas bei 2000 g erhalten. Die Extinktionsmessungen wurden mit einem Eppendorf-Fotometer mit dem Filter 578 durchgeführt. In Abänderung der von Born [4] beschriebenen Methode wurde die Durchmischung des plättchenreichen Plasmas mit Natrium-Oleat bzw. Natrium-Oleat und ADP durch Kippen der Gläschen und Küvetten ersetzt. In der Originalmethode [4] wird das plättchenreiche Plasma durch Rotation eines Stäbchens gerührt (stirring). Die infolge der verwendeten hohen Umdrehungszahl des Rührstäbchens von 1000 U/min verursachte mechanische Belastung der Thrombozyten ist gross genug, um einen Teil der Plättchen zu zerstören [3]. Die dabei freiwerdenden Substanzen, insbesondere ADP können selbst eine Aggregation der Thrombozyten auslösen. In einer Mitteilung von Gross *et al.* [14] wurde die Bornsche Originalmethode ebenfalls so verändert, dass keine mechanische Alteration der Thrombozyten mehr eintritt.

Nach Zusatz der einzelnen Substanzen zum plättchenreichen Plasma (PRP) wurden die Gläschen mit Parafilm verschlossen und zunächst 20mal gekippt. Die Extinktionen wurden dann in einminütigen Abständen gemessen, beginnend jeweils 60 sec nach Substanzzugabe. 15 sec vor jeder folgenden Ablesung wurde die Küvette wiederum 5mal gekippt. So konnte bei ausreichender Durchmischung des Plasmas eine Sedimentation der Thrombozytenaggregate vermieden werden [8]. Parallel zu jedem untersuchten plättchenreichen Plasma wurde ein Kontrollplasma des gleichen Probanden mitgeführt, dem anstelle der Testsubstanzen ein gleiches Volumen physiologischer Kochsalzlösung zugegeben wurde.

Alle Versuche wurden bei Zimmertemperatur (20 bis 22° C) und unmittelbar im Anschluss an die Blutentnahmen vorgenommen. Sämtliche Zusätze zum Plasma erfolgten in Form von 0,1 ml Lösung der entsprechenden Substanzen in physiologischer Kochsalzlösung. Dabei wurden die Konzentrationen dieser Lösungen so gewählt, dass 0,1 ml ad 5 ml Plasma die angegebenen Konzentrationen, bezogen auf Plasma, ergaben. Natrium-Oleat (Riedel-de-Haen AG, Seelze) wurde in 0,9% NaCl gelöst. Als aggregationsauslösende Substanz wurde das Trinatriumsalz des Adenosin-5-Diphosphats der Fa. C.F. Boehringer und Söhne, Mannheim, in einer Endkonzentration von 2×10^{-4} M/l verwendet. Die Lösung wurde täglich neu hergestellt. Alle Geräte, die mit Blut oder Plasma in Berührung kamen, wurden nach den üblichen Vorschriften silikonisiert. Die Genauigkeit der Extinktionsmethode ergab bei Wiederholungsmessungen eine maximale Abweichung von nur 2,63% [8]. Ein Schema der Versuchsabläufe zeigt Abb. 1

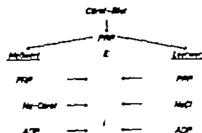


Abb. 1. Schema des Ablaufs der Versuche.

Ergebnisse

1 Einfluss von Natrium-Oleat auf die Aggregation von Thrombozyten in plättchenreichem Plasma

Jeweils 8 plättchenreiche Plasmen wurden auf 10 20 40 80 und 160 mg% Natrium-Oleat Konzentration gebracht Parallel zu jedem Messwert wurde ein Kontrollversuch mit physiologischer Kochsalzlösung mitgeführt. Die Beobachtungsdauer betrug 5 Minuten nach Zugabe von Natrium-Oleat bzw physiologischer Kochsalzlösung zu plättchenreichem Plasma. In Vorversuchen [8] hat es sich ergeben, dass eine längere Beobachtungsdauer nicht erforderlich ist.

Die Abb 2 zeigt den Extinktionsverlauf 8 verschiedener plättchenreicher Plasmen nach Natrium-Oleat Zusatz von 20 mg%. Die Extinktionskurven der Kontrollversuche mit physiologischer Kochsalzlösung verliefen deckungsgleich mit den eingezeichneten Kurven. Wie in allen folgenden Abbildungen sind auch hier die Extinktionen der plättchenreichen Plasmen abzüglich der Extinktionswerte der jeweiligen plättchenfreien Plasmen aufgezeichnet worden. Die unterschiedlichen Ausgangshöhen der Kurven sind Ausdruck der in den verschiedenen Plasmen der einzelnen Probanden erhaltenen verschiedenen Anzahl von Blutplättchen. Aus der Abb. 2 ist ersichtlich, dass nach Zugabe von 20 mg% Natrium-Oleat zu plättchenreichem Zitratplasma keine Extintionsveränderung gegenüber den entsprechenden NaCl-Kontrollversuchen erfolgte Analog dazu verliefen auch die Untersuchungen mit 10 40, 80 und 160 mg% Natrium Oleat. In keinem der insgesamt 40 untersuchten plättchenreichen

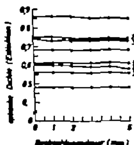


Abb. 2. Die Wirkung von Natrium-Oleat (20 mg%) auf die Aggregation der Thrombozyten in PRP Die Extinktionskurven der NaCl-Kontrollversuche verliefen deckungsgleich mit den eingezeichneten Kurven. Die Ziffern 1–8 bezeichnen die verschiedenen Plasmen.

Das Blut wurde in silikonierten Gläschen aufgefangen, in denen so viel Natrium-Zitrat-Lösung vorlag, dass eine Endkonzentration von 3,8% im Blut erreicht wurde. Plättchenreiches Plasma wurde durch 20 min dauerndes Zentrifugieren des Blutes bei 500 g, plättchenfreies Plasma durch 20minütiges Zentrifugieren des plättchenreichen Plasmas bei 2000 g erhalten. Die Extinktionsmessungen wurden mit einem Eppendorf-Fotometer mit dem Filter 578 durchgeführt. In Abänderung der von Born [4] beschriebenen Methode wurde die Durchmischung des plättchenreichen Plasmas mit Natrium-Oleat bzw. Natrium-Oleat und ADP durch Kippen der Gläschen und Küvetten ersetzt. In der Originalmethode [4] wird das plättchenreiche Plasma durch Rotation eines Stäbchens gerührt (stirring). Die infolge der verwendeten hohen Umdrehungszahl des Rührstäbchens von 1000 U/min verursachte mechanische Belastung der Thrombozyten ist gross genug, um einen Teil der Plättchen zu zerstören [3]. Die dabei freiwerdenden Substanzen, insbesondere ADP, können selbst eine Aggregation der Thrombozyten auslösen. In einer Mitteilung von Gross *et al.* [14] wurde die Bornsche Originalmethode ebenfalls so verändert, dass keine mechanische Alteration der Thrombozyten mehr eintritt.

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Abb. 1. Schema des Ablaufs der Versuche.

5 min nach ADP Zusatz ist im allgemeinen das Maximum der Thrombozytenaggregation erreicht (Abb. 3) So wie am Beispiel dieses Einzelversuches mit einer Natrium-Oleat Konzentration von 20 mg% gegenüber der Kontrolle mit NaCl-Zusatz zu plättchenreichem Plasma dargestellt (Abb 3) wurden für jeden Einzelversuch entsprechende Kurven aufgezeichnet, so dass für jede Natrium-Oleat Konzentration jeweils 8 Kurvenpaare erhalten wurden. Aus diesen 8 Kurvenpaaren wurden Mittelwerte errechnet und graphisch auf getragen (Abb. 4 und 5)

Die Abb 4 zeigt die arithmetischen Mittelwerte der Extinktionen 8 verschiedener plättchenreicher Plasmen nach ADP Zugabe.

Bei Zusatz von Natrium-Oleat in einer Konzentration von 10 mg lagen die Extinktionswerte nach ADP Zugabe geringgradig höher als bei den Kontrollversuchen, was einer Verringerung der Thrombozytenaggregation entspricht.

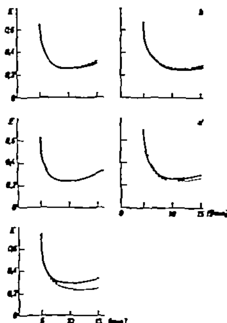


Abb. 4 Kurven der arithmetischen Mittel der Extinktionswerte (E) je 8 verschiedener PRP nach ADP-Zugabe (2×10^{-4} M/l). —— Plättchenreiche Plasmen mit Natrium-Oleat in einer Konzentration von 10 mg% ( ) 20 mg% (b) 40 mg% (c), 80 mg% (d), 160 mg% (e), —— Vergleichsproben der gleichen Plasmen, die NaCl anstelle von Natrium-Oleat enthalten. Natrium-Oleat bzw NaCl wurden bei t = 0 min eingepipettiert, ADP bei t = 5 min.

Plasmen wurde durch Natrium-Oleat Zusatz eine Extinktionsänderung gefunden. Auch mikroskopisch waren nach Natrium-Oleat Zusatz bis 80 mg% keine Thrombozytenaggregate nachzuweisen lediglich bei einer Plasmakonzentration von 160 mg% Natrium-Oleat waren vereinzelt Thrombozytenaggregate zu finden.

2. Einfluss verschiedener Natrium-Oleat Konzentrationen auf die ADP-induzierte Thrombozytenaggregation

Jeweils 8 plättchenreiche Plasmen wurden auf 10 20 40 80 und 160 mg% Natrium-Oleat Konzentration gebracht. 5 min nach dem Zusatz von Natrium-Oleat zu den plättchenreichen Plasmen wurde ADP Lösung zupipettiert. Wie sich aus Vorversuchen ergab, ist es unerheblich, ob zunächst ADP und dann Natrium-Oleat dem plättchenreichen Plasma zugesetzt wird oder ob umgekehrt vorgegangen wird [8] Die Wirkung von Natrium-Oleat auf eine ADP-induzierte Aggregation von Thrombozyten ist aus dem Vergleich zu den in jedem Fall mitgeführten Kontrollversuchen (Zusatz von physiologischer Kochsalzlösung anstelle von Natrium-Oleat Lösung) ersichtlich. Es sollte so nachgewiesen werden, ob eine ADP-induzierte Thrombozytenaggregation durch Zusatz von Natrium-Oleat in verschiedenen Plasmakonzentrationen verstärkt oder vermindert wird.

Wie Abb 3 zeigt, kommt es innerhalb der ersten beiden Minuten nach Zusatz von ADP zu plättchenreichem Plasma zu dem bekannten Extinktionsabfall (Absinken der optischen Dichte) Gleichzeitig lassen sich schon makroskopisch Thrombozytenaggregate nachweisen.



Abb. 3 Änderung der Extinktion eines PRP nach ADP-Zusatz (2 × 10⁻⁶ M/l). ●—●—● PRP mit Natrium-Oleat in einer Konzentration von 20 mg%. ○—○—○ PRP mit NaCl anstelle von Natrium-Oleat. Natrium-Oleat bzw NaCl wurden zur Zeit 0 einpipettiert, ADP 5 min später

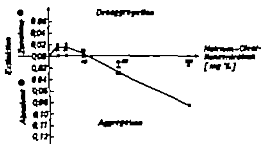


Abb 5. Extinktionsdifferenzen zwischen Mess- (Natrium-Oleat-) und Kontroll- (NaCl-) Plasmen in Abhängigkeit von den geprüften Natrium-Oleat-Konzentrationen. Die schwarzen Quadrate geben die Mittelwert der Extinktionsdifferenzen an. Ordinate: Unterschiede der optischen Dichte zwischen Mess- und Kontrollplasmen, gemessen in Einheiten der Extinktionsskala des benutzten Gerätes.

tistische Signifikanz sowohl für die desaggregierende Wirkung von Natrium-Oleat in Konzentrationen von 10 und 20 mg% als auch für die aggregierende Eigenschaft von Natrium-Oleat Konzentrationen über 40 mg% ist aus der Abb 5 deutlich ersichtlich (Zeichentest) eine weitergehende statistische Prüfung hatte sich deshalb erübrigt.

Diskussion

Der Ölsäure, als einer einfach ungesättigten C_{18}-Fettsäure, kommt deswegen besondere Bedeutung zu, weil sie von den im Plasma vorkommenden unveresterten Fettsäuren den höchsten Anteil ausmacht [19] In menschlichem Plasma, das bei einem pH von 7 4 eine grosse Pufferkapazität besitzt, sind die unveresterten Fettsäuren nahezu vollständig dissoziiert, da Fettsäuren als Essigsäure-analoge Carbonsäuren einen pK (wässerige Lösung) von 4 7 bis 5 0 aufweisen. Der Zusatz von Natrium-Oleat zu Blut in Konzentrationen bis 120 mg% verändert den pH des Blutes bzw Plasmas nicht [12] die Wirkungen auf Erythrozyten [13 12, 10] und Thrombozyten sind demnach dem Fettsäureanion zuzuschreiben.

Aus den vorliegenden Ergebnissen geht hervor dass Zusatz von Natrium-Oleat zu Plasma in Konzentrationen bis 40 mg/100 ml eine partielle Desaggregierung von Plättchenaggregaten bewirkt. Hohe Konzentrationen von Natrium-Oleat über 40 mg% verstärken demgegenüber die ADP induzierte Thrombozytenaggregation. Diese

Befunde stehen teilweise im Widerspruch zu den Ergebnissen anderer Autoren. Hierfür dürften an erster Stelle methodische Unterschiede verantwortlich sein. Von HOAK *et al.* [16] sowie MAHADEVAN *et al* [18] wurde nachgewiesen, dass Natrium-Oleat sowohl auf gewaschene Thrombozyten als auch auf Thrombozyten in plättchenreichem Plasma aggregationsfördernd wirkt. Relativ niedrige Natrium-Oleat Konzentrationen (bis 60 mg %) aggregierten nach weiteren Feststellungen jedoch lediglich gewaschene Thrombozyten, nicht aber Blutplättchen in Plasma [16] Die gleichen Verfasser konnten weiterhin nachweisen dass die aggregierende Wirkung des Natrium-Oleates auf gewaschene Thrombozyten durch vorherigen Zusatz von ausreichend Albumin nahezu aufgehoben werden kann. ARDLIE *et al.* [1] fanden bei niedrigen Oleat Konzentrationen weder eine aggregierende Wirkung des Natrium-Oleates in plättchenreichem Plasma noch eine Beeinflussung der ADP-induzierten Plättchenaggregation. Die Anwesenheit von Serumalbumin ist offenbar bei *in vitro*-Untersuchungen über die Plättchenaggregation von entscheidender Bedeutung da dem Plasma zugesetzte Ölsäure bzw Natrium-Oleat nicht frei vorliegt, sondern an Albumin gebunden wird [11 16] Auch die unveresterten Fettsäuren des Plasmas *in vivo* sind an Albumin gebunden «freie» Ölsäure (ohne Bindung an Plasmaalbumin) kommt *in vivo* praktisch nicht vor Die fehlende aggregierende Wirkung von Natrium-Oleat in Konzentrationen unter 40 mg % auf im Plasma suspendierte Thrombozyten dürfte daher Folge einer Symplexbildung zwischen dem Albumin und dem Oleat Anion sein, so dass es nicht zu einer direkten Beeinflussung von freiem, nicht an Albumin gebundenem Natrium-Oleat auf die Blutplättchen kommt.

Unter diesen Gesichtspunkten sind auch die Ergebnisse von HOAK *et al* zu erklären, die das Auftreten von Thromben in den Lungen von Versuchstieren nach intravenösen Injektionen von Albuminlösungen mit hohen Natrium-Oleat Konzentrationen beschrieben [15 16] Möglicherweise bestand bei der grossen Menge von zugesetztem Natrium-Oleat zur injizierten Albumin-Lösung keine quantitative Bindung der Fettsäureanionen an das Albumin so dass freies, nicht gebundenes Natrium-Oleat direkt auf die Plättchen einwirken konnte. Ähnliche Vorbehalte wie für die Arbeiten von HOAK *et al* gelten auch für die Ergebnisse von ZBINDEN [20] und DAY [6] die nach Natrium Oleat Infusionen bei Tieren eine Thrombopenie beobachteten

Intravital erfolgt der Anstieg der Konzentration der unveresterten Fettsäuren bei Stresszuständen beim Rauchen und während

des Hungers relativ langsam und auf das gesamte Plasmavolumen verteilt. Bei der therapeutischen Anwendung von Heparin und Heparinoiden kommt es zu einem relativ schnellen Anstieg der unveresterten Fettsäuren im Plasma, wobei die Fettsäuren quantitativ an Albumin gebunden sind. Die durch Heparininjektionen ausgelöste Erhöhung der Konzentration an unveresterten Fettsäuren übersteigt jedoch im allgemeinen nicht 40 mg% und liegt demnach in einem Konzentrationsbereich, der nach den vorliegenden Ergebnissen eine leichte desaggregierende Wirkung auf Thrombozytenaggregate aufweist.

Die partiell desaggregierende Wirkung von Natrium-Oleat in Konzentrationen bis *40 mg% auf ADP* induzierte Thrombozytenaggregate zeigt eine auffallende Parallelität zu der früher beschriebenen desaggregierenden Wirkung von Natrium-Oleat auf Erythrozytenaggregate [13] Während jedoch für Erythrozytenaggregate bei höheren Konzentrationen die desaggregierende Wirkung zunimmt, ist dies bei Thrombozytenaggregaten nicht der Fall (s. Abb 5) Die Ursache der thrombozytendesaggregierenden Wirkung von Natrium-Oleat in geringen Konzentrationen und der aggregationsfördernde Effekt von hohen Konzentrationen von Natrium-Oleat konnten bisher nicht gefunden werden. Bei Erythrozytenaggregaten erfolgt die Desaggregation über eine Abrundung der Zellen (bikonvexe Erythrozyten) Der Nachweis eines entsprechenden oder ähnlichen Mechanismus für die Thrombozytendesaggregation nach Zusatz geringer Konzentrationen von Natrium-Oleat gelang mit den üblichen Methoden (Phasenkontrastmikroskop) nicht.

Der Hemmung der Aggregation der Thrombozyten kommt im Zusammenhang mit der Prophylaxe und der Therapie thromboembolischer Erkrankungen und möglicherweise auch bestimmter Schockzustände eine besondere Bedeutung zu, da die Zusammenlagerung der Thrombozyten einen maßgeblichen pathogenetischen Faktor dieser Erkrankungen darstellt. Nach Deutsch [7] Böhle *et al.* [2] Zweifler [21] und anderen Autoren ist weiter die Möglichkeit zu diskutieren, dass arteriosklerotischen Frühveränderungen über eine Minderung der Thrombozytenaggregation zu begegnen ist. Die vorliegenden Untersuchungen mit Natrium-Oleat ergaben eine leicht desaggregierende Wirkung auf Thrombozytenaggregate bei physiologisch denkbaren Konzentrationen. Den Salzen anderer unveresterter Fettsäuren (z. B. Palmitinsäure, Stearinsäure) wird eine aggregationsfördernde Wirkung auf Thrombozyten nachgesagt [5 16 17 18] Dabei wurden aller

dings relativ hohe Fettsäurekonzentrationen untersucht. Angesichts der Tatsache, dass Natrium-Oleat ein bivalentes Verhalten in Beziehung auf die Thrombozytenaggregation zeigt, erscheinen entsprechende Untersuchungen mit niedrigen Konzentrationen anderer Fettsäuren angezeigt.

Zusammenfassung

Mit einer Modifikation der Bornschen Methode zur Messung der Thrombozytenaggregation wurde die Wirkung verschiedener Konzentrationen von Natrium-Oleat sowohl auf Thrombozyten als auch auf ADP-induziert Thrombozytenaggregate untersucht. Es ergab sich, dass Natrium-Oleat Konzentrationen bis 80 mg/100 ml Plasma keine Aggregation der Thrombozyten verursacht. Die Wirkung von Natrium-Oleat auf bereits bestehende Thrombozytenaggregate ist verschieden. Während niedrige, physiologisch denkbare Konzentrationen bei 40 mg% eine leicht Desaggretion von Thrombozytenaggregaten bewirken, führen Natrium-Oleat Konzentrationen über 40 mg% zu einer Verstärkung der bereits vorhandenen Thrombozytenaggregation.

Summary

Aggregation of platelets has been studied *in vitro* by a modification of Born's method. It could be shown, that addition of sodium-oleate to platelet rich human plasma does not cause platelet aggregation in concentrations up to 80 mg%. When platelets in plasma had been aggregated by ADP before, the addition of sodium oleate in concentrations up to 40 mg% leads to a partial desaggregation of the platelet aggregates, while higher concentrations enhance the aggregation of platelets.

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The Effect of Adrenergic Blockers on the Action of Adrenaline on the Factor VIII Activity in the Plasma

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The observation of Ingram [4] that the intravenous infusion of adrenaline brings about a considerable elevation of the factor VIII activity in the plasma of normal subjects, was confirmed by other workers [3, 7]. According to Ingram isopropylnoradrenaline and noradrenaline have no similar effect. The mechanism of this action of adrenaline remains unexplained.

Ahlquist [1] has first shown that the various actions of the sympathomimetic drugs are mediated through two special receptor groups, the α and β receptors. Adrenaline acts on both types of receptors, noradrenaline on the alpha, whilst isopropylnoradrenaline almost exclusively on the β receptors. Various agents block selectively the action on the α or the β receptors. Thus, tolazoline, phentolamine, benzodioxane, dibenamine and phenoxybenzamine are α-adrenergic blocking agents, whilst dichloroisoprenaline, pronethalol and propranolol are β adrenergic blocking agents.

The present paper describes investigations on the effect of α- or β-adrenergic blocking agents on the action of the adrenaline on the factor VIII activity in the plasma.

Material and Methods

Experiment I. Ten normal adult males were used. ( ) Adrenaline was administered i.v. (160 μg in 10 ml of 5% glucose in 10 min). Before and 5 min after the end of the infusion venous blood was collected. (b) Two days later phentolamine (Regitine ®), an α adrenergic blocker was given by i. infusion (10 mg in 200 ml of 5% glucose in 30 min). On the 20th minute of the phentolamine infusion adrenaline was administered as described above in

10 min through vein of the other arm. Five min after the end of the infusion venous blood was collected.

Experiment II. Ten normal adult males were used. ( ) Adrenaline was administered as described in part ( ) of experiment I. Before and 5 min after the end of the infusion venous blood was collected. (b) Two days later and for three consecutive days the subjects received orally propranolol (Inderal ®) 60 mg daily divided into 4 six-hourly doses. On the fourth day immediately after the morning propranolol dose adrenaline was injected as described in part ( ) of experiment I. Five min after the end of the adrenaline infusion venous blood was collected.

Assay of factor VIII. Samples of 9 ml of venous blood taken with siliconized needles and glass syringes were mixed with 1 ml of trisodium citrate ($Na_3C_6H_5 \cdot 2H_2O$ 3.8 g/100 ml) in siliconized glass test tube. After centrifugation for 15 min at 300 rpm the plasma factor VIII concentration was assayed immediately by the method described by BIGGS and MACFARLANE [2]. As reference standard mixed pool of plasma from 10 normal donors stored in 1 ml amounts at —30° C was used.

Results

As the tables I and II show neither Regitine® and α-adrenergic blocker nor propranolol, a β-adrenergic blocker inhibit the effect of adrenaline on the factor VIII activity of plasma.

The present experiments support the view that the action of adrenaline on the factor VIII activity of the plasma is not mediated through the special receptor groups, as special adrenergic blockers do not inhibit this action. Thus, the mechanism of the rise of factor VIII activity in the plasma produced by adrenaline remains obscure.

Table I. The effect of Regitine® on the action of adrenaline on the factor VIII activity

Experiment	Factor VIII activity in the plasma (% of normal) before adrenaline	after administration of adrenaline	after administration of adrenaline + regitine
1	98	185	197
2	102	212	208
3	114	265	253
4	94	206	220
5	108	228	222
6	115	301	307
7	96	183	194
8	101	216	209
9	100	192	202
10	107	227	220

The figures represent the mean value of 3 determinations.

Table II The effect of propranolol on the action of adrenaline on the factor VIII activity

Experiment	Factor VIII activity in the plasma (% of normal)		
	before	after administration of	
	adrenaline	adrenaline	adrenaline + propranolol
1	96	214	220
2	102	176	190
3	112	235	228
4	98	206	210
5	98	197	191
6	102	290	282
7	110	198	211
8	98	265	262
9	108	179	188
10	102	221	214

The figures represent the mean value of 5 determinations.

Discussion

The present experiments show that neither phentolamine, an α-adrenergic blocker nor propranolol, a β-adrenergic blocker inhibit the effect of adrenaline on the factor VIII activity. These results are in agreement with the findings of McCLURE *et al* [6] that α-adrenergic blockade with phentolamine did not prevent elevation of factor VIII after adrenaline infusion. On the other hand, INGRAM [5] found that pronethalol a β-adrenergic blocker inhibits the elevation of factor VIII brought about by adrenaline infusion. No plausible explanation for the discrepancy of these findings can be given.

Acknowledgement

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Summary

Phentolamine an α-adrenergic blocker and propranolol, β-adrenergic blocker do not inhibit the effect of adrenaline on the factor VIII activity in the plasma.

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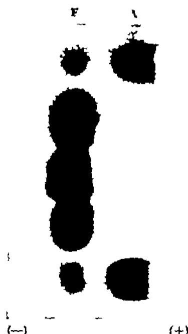


Fig. 2. Agar gel electrophoresis in citrat buffer pH 6.0. At the top and the bottom: haemoglobin of the parents of the propositus. In the center: haemoglobin of the propositus and of the other two children, homozygous carriers of βδ-thalassaemia. Total absence in these three subjects of haemoglobin A_1 and presence of haemoglobin F only

S. Concetto (II 1 table I, fig. 1) the *father of the propositus* was healthy subject which showed haematologic picture typical of microcythaemia and haemoglobin pattern with an elevated level of haemoglobin F (10%) and normal level of haemoglobin A_2 (2.02%) Acid elution test showed quite an heterogeneous intracellular distribution of haemoglobin F

A. Domenica (II 2, table I fig. 1) the *mother of the propositus* and first cousin of the father was healthy woman with a haematologic microcythaemic picture and a haemoglobin pattern with levated haemoglobin F (7.50%) normal haemoglobin A_2 (2.30%) and heterogeneous intracellular distribution of haemoglobin F

A. Gregorio (I 1 table I, fig 1) *paternal grandfather of the propositus*, was healthy microcythaemic subject with the same haemoglobin characters of the son (haemoglobin F 9.55% haemoglobin A_2 1.87%) and the same heterogeneous aspect of the ... cellular distribution of haemoglobin F

Discussion

Both the parents and the paternal grandfather of the patient observed presented the haematologic abnormalities typical of mi cythaemia (or thalassaemia) with high level of haemoglobin F and heterogeneous erythrocytic distribution of haemoglobin F These data

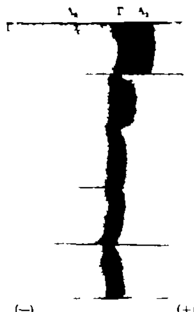


Fig. 3. Cellulose acetate electrophoresis, glycine buffer pH 8.8. From the top to the bottom the father of the propositus Cooley disease patient the three sisters, homozygous carriers of βδ-thalassaemia. Total absence of haemoglobin A_2 and haemoglobin A_1 in the three homozygous subjects.

are typical of F microcythaemia. The three children show a microcythaemic haematologic picture and their haemoglobin is only haemoglobin F These data suggest that the children are homozygous for F microcythaemia. From the clinical point of view the propositus was the only one to present a slight anaemia with moderate hepatosplenomegaly the other two sisters are in good health, and have no history of illness.

This is the third family reported in the literature in which cases of homozygosity for F microcythaemia have been found. In the first family [20] the propositus was a 31 year-old woman with slight pallor and marked hepato-splenomegaly in the second case [22] the condition was discovered accidentally in a 9-year-old boy Thus, all these observations agree in showing that the homozygous condition for F microcythaemia causes the subjects to be far less severely affected than the homozygous carriers of A_2-microcythaemia.

The same tendency towards a milder clinical picture is also found in the patients with double heterozygosity for A_2- and F microcythaemia.

The cases so far observed include 8 patients with severe Cooley's disease [1 8, 16 17] and at least 7 other patients with a much less serious disease (microcytic constitutional anaemia or thalassaemia intermedia) [5 17–19] In another series [12, 22] most of the 21 patients were moderately affected

These data show that the F microcythaemia is a microcythaemia variant which interacts with classical A_2 microcythaemia and, besides this, they offer the documentation of new variants of genetic basis of the thalassaemia intermedia that is the homozygosity for βδ-microcythaemia with high F and the double heterozygosity for this microcythaemia variant and classical A_2 microcythaemia

The present patients and the two of the literature had haemoglobin F only Homozygosity for F microcythaemia is thus the third homozygous condition, so far described, in which there is a complete absence of haemoglobin A_1 and haemoglobin A_2, the other two being homozygous condition for haemoglobin Lepore and homozygous condition for hereditary persistence of foetal haemoglobin and the second condition in which haemoglobin F only is found (the other being homozygosity for high F gene) This proves that in the three families so far described the βδ-microcythaemia genes completely suppress β- and δ-chain synthesis.

There are, till now only few cases which can supply informative data on the activity of the structural genes β and δ in this variant of microcythaemia The three present and the two preceding homozygotes [10 21 22] and the double heterozygote for B_2 haemoglobin and βδ-microcythaemia [14] show a complete inactivity of locus δ As for locus β in the 5 homozygotes for F microcythaemia and in the 2 double heterozygotes for haemoglobin S and F microcythaemia [4 6] such locus has proved to be completely inactive in 6 cases and partially functioning in 1 case [4] It would appear that in F microcythaemia *likewise in A_2-microcythaemia, locus β is in most instances suppressed* and rarely partially functioning as for locus δ on the contrary the observations are not sufficient to prove a constant and complete inactivity A larger number of cases is necessary for the final interpretation of the genic effects of this microcythaemic mutation

In βδ-microcythaemia with high F locus γ appears affected in a characteristic way there is, in the heterozygote state an evident hyperactivity which has such a constant uniformity as to appear genetically determined. On the contrary the resulting haemoglobin F is irregularly distributed in the erythrocytes – like in the other forms of

microcythaemia – a fact that would suggest a compensatory mechanism. Therefore the compensatory hyperactivity of locus γ is typically evident in the F microcythaemia heterozygote, but not in the A_2-microcythaemia heterozygote. Moreover in the $\beta\delta$-microcythaemia homozygote the hyperactivity of locus γ often reaches a much higher functional level than in the classical β-microcythaemia with high A_2 homozygote thus considerably reducing the severity of clinical manifestations.

This consideration and the equality of the haemoglobin picture of homozygous carriers of F microcythaemia and of hereditary persistence of haemoglobin F brings to the fore the problem of relationships between microcythaemia and hereditary persistence of haemoglobin F. The similarity between the two conditions is highly suggestive. It is however clear that the two conditions cannot be based on the same genetic alteration. While in homozygous F microcythaemia state the degree of haemoglobinization is always below the normal range (so that the haematological picture of microcythaemia is always present) in homozygous state for high haemoglobin F gene the normal haemoglobin level reveals that loci γ are both fully functioning.

Therefore in F-microcythaemia any future hypothesis on the basic genetic mechanism shall have to take into account not only the total or partial suppression of the products of the two adjacent structural genes β and δ but also the partial and characteristic persistence of activity of structural gene γ which is constant and typical in this microcythaemia variant.

Summary

In [illegible] family of Sicilian origin the parents, who are first cousin to each other, are both carriers of $\beta\delta$-thalassaemia (or microcythaemia) with high haemoglobin F (F-thalassaemia) and their three children are homozygous carriers of F-thalassaemia. Clinically only the propositus shows [illegible] slight anaemia and [illegible] moderate hepato-splenomegaly. His two sisters are apparently healthy. As the two preceding observations of literature, this family proves that the activity of normal β and δ genes is completely suppressed in F-thalassaemia, and that the homozygous condition produces [illegible] much less severe disease than the homozygosity for β-thalassaemia with high haemoglobin A_2 genes.

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Complete Remission in Chronic Lymphocytic Leukemia

Report of Case

P Pardo, E. Simó M Ribas-Mundó and P Farreras

Patients with chronic lymphocytic leukemia have usually a long survival either spontaneously or with treatment, but lymphocytic infiltration of the bone marrow and blood lymphocytosis persist in the majority of cases throughout the course of the disease.

We report a patient with chronic lymphocytic leukemia in whom complete clinical and hematological remission, proven by even cytochemical and lymphographic techniques, developed after therapy with cyclophosphamide and prednisone. After the complete remission it was impossible to find any feature which let us suspect the persistence of chronic lymphocytic leukemia. As this is an unusual case not often reported in the literature we think it might be worth to report it.

Case Report

A 63 years old, married, railroad employé, born in Peñalba (Huesca) Spain. Family history was not significant. At the age of 24 he suffered pleuritis of unknown etiology At the age of 35 ocular surgery for strabismus. Since then he has complaint of frequent upper respiratory infections.

His present illness began at the age of 63 (February 1961) with bronchitis, progressive lassitude and acute and severe pain of the left eye followed by amaurosis. A diagnosis of glaucoma was made At this time an hemogram revealed no anemia 15 770 leukocytes with 96 lymphocytes and numerous Gumprecht cells.

Physical examination revealed an apparently healthy man with amaurosis of the left eye and generalized lymphadenopathy of medium size in cervical, axillary and inguinal regions.

Laboratory analysis (24 II.61) E.S.R. 11 mm at the first hour red blood cell count 4,565,000, hemoglobin 13.6 g%, total leukocyte count 15,770 with juvenile forms 1 %

segmented neutrophils 3%, lymphocytes 96% and many Gumprecht cells. Myelogram hypoplastic erytropoiesis of about 8% of the total cellularity 80% of infiltrating mature lymphocytes and some lymphoblasts, scattered myelocytes and metamyelocytes. No megakaryocytes. Total proteins 3.6 g%, albumine 3.08 g%, globuline 1.24 g%. Uric acid 8.55 mg%. Platelet count 230,000 Due to the good general condition of the patient no specific treatment was given.

Three months later (3 VI.61) the leukocyte count rose to 147,070 and an enlarged spleen was detected 4 cm under the left costal margin. Treatment was started with 150 mg cyclophosphamide and 10 mg prednisone per os daily and was given for 3 months. I September 1961 the spleen was not palpable and the leukocyt count had dropped to 14,080. Treatment was reduced to 200 mg cyclophosphamide i.m. weekly and 5 mg prednisone per os daily

During this time he suffered from frequent upper respiratory infections. A basocellular epithelioma infiltrating the left preauricular region and spinocellular epithelioma on the right temporal region also developed.

After year of treatment (8.V 62) with cyclophosphamide and prednisone the peripheral leukocyte count was 11,500 with 57% lymphocytes. The bone marrow aspirat revealed 20% lymphocytes. On physical examination the liver was still palpable 3 cm below the right costal margin and axillary lymph nodes were still felt. The same treatment was prescribed.

I May 1964, three years after beginning of treatment, the patient was in good condition. On physical examination only the liver was palpable 2 cm below the costal margin neither splenomegaly nor lymphadenopathies were detected. Red blood cell count was 5,200,000, Hb 14.7 g% and the leukocyte count was 8,250 with 22% lymphocytes. Trea ment with cyclophosphamide 50 mg and prednisone 5 mg per os every other day was continued. I December 1964 the liver was no longer palpable. Analysis of peripheral blood was normal and treatment was stopped.

I July 1966 physical examination was normal. The erythrocyte count was 5,300,000 with 16.1 g% Hb, the leukocyt count was 7,370 with 36% lymphocytes; only 15% of these lymphocytes presented PAS reaction. The bone marrow aspiration was normal with 5% lymphocytes.

I July 1967 the patient was asymptomatic and physical examination was entirely normal. The hematological data remained within the normal limits. Less than 10% of the peripheral lymphocytes presented PAS reaction. The bone marrow aspiration revealed normal erythro- and leukopoiesis, presence of megakaryocytes and 12% of lymphocytes. The lymphography in July 1967 failed to reveal enlarged retroperitoneal lymph nodes.

I March 1968 the patient is still in good clinical condition. Neither hepato-splenomegaly nor lymphadenopathies are palpable. Nevertheless, peripheral blood leukocyte count has risen to 13,560 with 52% lymphocytes. Bone marrow aspiration reveals 30% lymphocytic infiltration. Treatment with cyclophosphamide 50 mg and prednisone 5 mg every other day is reinstaured.

Discussion

Chronic lymphocytic leukemia has always been considered the less malignant of all types of leukemia. Patients with long survival are common. Our case, however has remained in complete remission for at least 3 years with no clinical or laboratory features of chronic lymphocytic leukemia.

In the available literature we have found very few cases were the complete remission of chronic lymphocytic leukemia had been satis-

factorily proven REICH [1] published in 1959 a case of complete remission after radiotherapy of the spleen (1 700 r) and blood transfusions. The patient persisted on complete remission for 5 years. DURANT and FINKBEINER [2] published in 1964 a revision of 250 cases of chronic lymphocytic leukemia, lymphosarcoma and leuko-lymphosarcoma. These authors comment the difficulties encountered in some cases to differenciate chronic lymphocytic leukemia and lymphosarcoma. From the 250 cases only one achieved complete remission during 3 years after treatment with prednisone and TEM. In 1955 a case of remission in a female treated with radiotherapy was published by SCHOTT [3]. However there is no mention of the myelogram during the remission phase. Recently TIN HAN *et al.* [4] have described 6 cases of complete remission among 202 patients with chronic lymphocytic leukemia and leukolymphosarcoma. The lymphocytic infiltration in bone marrow of these cases during remission ranged however between 15% and 20%, which is over the upper limit for normal adults.

In our case the diagnosis of chronic lymphocytic leukemia seems unequivocal for he had generalized adenopathy, liver and spleen enlargement, white cell count of 147 000 with 96% lymphocytes and many Gumprecht cells, bone marrow infiltration with mature lymphocytes and some lymphoblasts. The leukocytosis of 13,560 with 52% lymphocytes and the 30% lymphocytic bone marrow infiltration after 3 years of clinical and hematological remission confirms the diagnosis of chronic lymphocytic leukemia.

We consider a complete remission to be present when there are no clinical or laboratory signs which could detect the disease. These criteria are consistent with the published criteria of BISEL [5] for complete remission of acute leukemia. We suggest two further criteria to accept the complete remission of chronic lymphocytic leukemia: (1) The PAS reaction of the peripheral lymphocytes should be positive in less than 20% as is in normal subjects; (2) the lymphography should be normal.

In our patient complete remission during 3 years can be proved by the following data: (1) No clinical symptoms of disease; (2) no palpable adenomegaly, no hepatoesplenomegaly; (3) red blood cell count 5 300 000, Hb 102%, white blood cell count 7 370 with 36% lymphocytes; (4) the lymphocyte content of the bone marrow is less than 15%; (5) less than 20% of the peripheral lymphocytes show a PAS reaction; (6) the lymphography is normal.

The maintenance dose of cyclophosphamide and prednisone even when the patient was in good condition has probably played a role in the evolution of this patient, but as Tin Han *et al* [4] comment, factors other than treatment might mediate in such cases. In accordance with these authors it is also possible that the frequency of complete remission in chronic lymphocytic leukemia might be increased significantly if patients were treated earlier and with a maintenance dosage of proven effective drugs in a long term therapy

At the Department of Hematology of the University of Barcelona we have only seen this single case of complete remission among 68 cases of chronic lymphocytic leukemia in a period of 10 years.

Summary

The authors report case of chronic lymphocytic leukemia which attained clinical and hematological remission during 3 years after treatment for 3 years with cyclophosphamide and prednisone. A review of the literature has revealed very few cases of complete remission in chronic lymphocytic leukemia. The authors suggest two further criteria of complete remission in chronic lymphocytic leukemia the PAS peripheral lymphocytes should not exceed 20% and the lymphography should be normal.

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Acute Erythromyelosis after Benzene Poisoning

C. Rozman S. Woessner and J. Saez-Serrania

In 1965 we published 20 cases of aplastic anemia induced by chronic benzene poisoning [1] Malignant neoplastic disorders of the bone marrow acute leukemia or less often chronic leukemia have been found in patients with prolonged exposure to benzene containing environments [2, 3] There are more than 100 of such cases referred to in the literature [3] Rare cases of erythromyelosis (erythremia in its pure form or as erythroleukemia) have been found associated with long contacts with benzene the latter form being more common than the former [3–13] The rarity of this cases has prompted us to report a patient with acute erythromyelosis after chronic benzene poisoning

Case Report

A 58-year-old varnisher was seen in January 1968, with previous history of working during 30 years in closed environment rich in benzene

In November 1967 after a short 'flu' with low fever the patient noticed onset of dyspnea after effort, lassitude and paleness. A blood count performed in early January showed normochromic anemia with 2,500,000 RBC/mm³ On examination hepatosplenomegaly was found. Chest roentgenogram was normal. In two separated occasions 500 ml of fresh blood were given, without improvement in the patient condition.

A hematological study perfomed on the 15th of February showed the following data RBC 1 480,000/mm³ hemoglobin 25 Hb: 27 pg Reticulocytes 1,500 mm³ Platelets 11,000 mm³ Leukocytes 11,000 mm³ myeloblasts 1 %, promyelocytes 5%, neutrophilic myelocytes %, metamyelocytes 7 %, young neutrophils 24 %, segmented neutrophils 41 %, lymphocytes 21 %, monocytes 1 5 erythroblasts per 100 Very marked anisopoikilocytosis some erythrocytes with crenated edges.

Bone marrow Proerythroblasts 1 4 %, basophil erythroblasts 40.0%, polychromatophil erythroblasts 19.0%, ortochromatic erythroblasts 17.2%, myeloblasts 0.8 %, promyelocytes 5.8%, metamyelocytes 1.2%, reticulum cells 4.6 %. Marked morphological changes of the erythroblasts were found mainly in the nuclei. Giant polyploid forms, diplo-erythroblasts, atypical mitoses and in some cells cytoplasmatic vacuoles were seen fig 1 3 There were

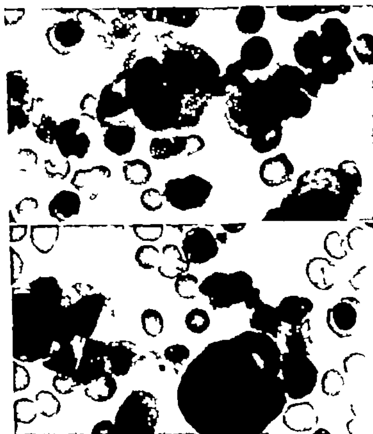


Fig 1 Giant and small polyploid erythroblasts with 4 and 5 nuclei.

no atypias in the white cell series. The number of megakaryocytes was scarce. Most erythroblasts showed an intense diffuse PAS-positivity of the cytoplasm. Some erythroblasts had PAS-positive granules. Perls stain showed 78% sideroblasts without ring forms.

The course of the illness was growing worse, without response to the therapy. The patient died on the 24 of February of massive intestinal hemorrhagy.

Comment

Clinically and experimentally it has been shown that benzene exerts its toxicity upon the erythroblastic mitoses resulting in giant erythroblasts, polyploid mitoses and arrested mitoses in metaphase [11 12 14]. The finding of diploerythroblasts in marrow medular aplasia identifies benzene as the most probable causal agent [15].

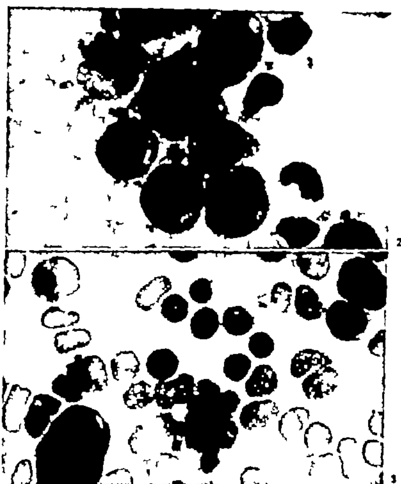


Fig 2. Group of young erythroblasts with vacuoles in the cytoplasm.

Fig 3. Three diploerythroblasts with nuclei arranged like coffee beans.

The above named anomalies and the history of prolonged exposure to benzene, have lead us to consider this substance as the causative agent. On the other hand, the other clinical and hematological findings are similar to the non benzene produced erythremias The ferrokinetic and erithrokinetic findings [1] and the PAS-positivity of the erythroblasts, as shown in our case, are similar in both types of erythremia There are a number of blood diseases, like thalassemia, iron-deficiency anemia and hemolytic disease of the newborn which show some PAS-positivity in the erythroblasts, but this is more intense in the

acute erythremia (Di Guglielmo's disease) and offers an important clue to its diagnosis [16]

Summary

A case of acute erythromyelosis is reported after 30 years exposure to benzene. Nuclear abnormalities were found in the erythroblasts, which showed marked PAS-positivity in their cytoplasm, like in other types of erythremia.

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Erratum

In der Arbeit SCHWAPPACH SCHÖNER „Zur Natur der stark basophilen Zellen in der Ductus thoracicus-Lymphe“ (Vol. 39, Nr. 3, 1968) wurden leider die beiden Abbildungen vertauscht.

Therapie der Leukosen und malignen Lymphome

In *Wien* findet vom *24.–26. März 1969* eine internationale Arbeitstagung über „Chemo- und Immunotherapie der Leukosen und malignen Lymphome“ statt. Leitung: Prof. Dr H. FLEISCHHACKER. Auskünfte: Doz. Dr A. STACHER, Ludwig-Boltzmann-Institut für Leukämieforschung und Hämatologie, *A-1140 Wien*, Heinrich Collin-Straße 30.

International Society of Blood Transfusion

At the meeting of the ISBT Executive Committee which took place in Moscow from the 25th February to the 1st March, it was decided that the Society could offer a few travel grants to help some young scientists to go to the XIIth International Congress of Blood Transfusion, Moscow, August 17–23, 1969. The total amount of money available for travel grants is $ 2000 which will be divided among a few candidates up to the age of 35 years, actively engaged in research, to assist them in paying for their travel expenses.

Applicants shall apply to Dr E. FREIESLEBEN, Blood Bank, Rigshospitalet, 9 Blegdamsvej, *2100 Copenhagen* (Denmark), furnishing the Committee with 4 copies, abstracts or reprints of the paper to be submitted, a complete curriculum vitae (including the age of the applicant) and a letter of recommendation from the applicant's chief or head of the research programme, as well as some information about the applicant's possibility of obtaining financial support from other sources. The applications should be sent before the 1st of February 1969.

Acta haemat. 40: 241–251 (1968)

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Measurements of Protein Synthesis by Lymphocytes *in vitro* in Diseases of the Lymphoid Tissues

J. W. M. LAWTON and I. J. FORBES

In earlier studies [8, 9] measurements showed reduced globulin synthesis by lymphocytes from peripheral blood in a variety of disease states. Of particular interest were low values in a few cases of chronic lymphatic leukaemia and macroglobulinaemia, suggesting that the reduced rate of immunoglobulin synthesis commonly found in these conditions [17] may be associated with measurable metabolic deficiency in the circulating lymphocytes.

Studies were therefore undertaken to extend these preliminary observations. The activity of lymphocytes was measured in medium containing autologous plasma and in plasma-free medium, to investigate the possibility that circulating substances may inhibit protein synthesis by lymphocytes in disease states. Parallel measurements were made of the effect of phytohaemagglutinin on protein synthesis. Lymphocytes were studied from normal subjects, and from patients with chronic lymphatic leukaemia, lymphoma (unclassified) and lymphosarcoma, macroglobulinaemia, multiple myeloma and infectious mononucleosis.

Materials and Methods

Normal subjects were healthy laboratory workers, medical practitioners, students, and blood donors. Diagnosis of chronic lymphatic leukaemia (CLL) was made on blood count of more than 9,000 lymphocytes/mm³ and the finding of non-follicular lymphocytic infiltration of the bone marrow [5]. Multiple myeloma was diagnosed by γ- or β-2A band of paraprotein on serum electrophoresis which was not precipitated from the serum by rivanol [15] associated with an infiltration of the bone marrow by plasma cells. I macroglobulinaemia the paraprotein was precipitated from the serum by rivanol, and was associated with

infiltration of the bone-marrow by abnormal cells of the lymphocyte series. The category 'lymphoma (unclassified) was made up of a small group of patients with lymphocytic infiltration of the bone marrow but without diagnostic changes in the lymph node blood or bone marrow. Diagnosis of lymphosarcoma was made from histological sections. Diagnosis of infectious mononucleosis was made in the presence of characteristic symptoms and signs, and the presence of atypical lymphocytes in the blood [6]. Heterophile agglutinins [12] were present in 6 cases, absent in 3, and not tested for in one case. The heterophile antibodies were not absorbed by guinea-pig kidney suspension [3] in 3 cases and were absorbed in one case.

Culture of lymphocytes. Venous blood, 40–50 ml where possible was collected into heparin (25 IU/ml) and dextran 0.3%. After the erythrocytes had sedimented for 1 h at 37°C, the supernatant plasma was taken and centrifuged at 800 *g* for 15 min. The sedimented leucocytes were resuspended in half the volume of plasma and incubated at 37°C for 1 h in a glass column packed with cotton wool. The lymphocytes were eluted from the column with the other half of autologous plasma, washed twice with BSS and suspended in Eagle's medium supplemented with non-essential amino acids [1]. From each blood sample 12 × 1.0 ml cultures were prepared in 4 groups, so that each determination was made in triplicate. The composition of the cultures is shown in table I. A small volume of the lymphocyte suspension was dispensed separately for the determination of the lymphocyte count (Coulter Counter Model D) and for making a smear for a differential leucocyte count. Cultures were incubated at 37° for 24 h then frozen.

Measurement of protein synthesis. Cultures were frozen and thawed three times and centrifuged at 10,000 *g* for 15 min to remove cell debris. To each plasma-free supernatant was added 0.25 ml of standard human pooled serum. The protein was precipitated in 5% trichloracetic acid, washed three times, dissolved in NH_4OH and plated at infinite thickness on 2 cm^2 planchettes for counting in an end-window gas-flow Geiger Müller counter.

Total protein concentration of plasma was estimated by the method of Wolfson *et al.* [20].

Calculation of results. Net counts per minute (CPM) were obtained by subtracting the CPM of protein in cultures containing puromycin 60 µg/ml. These samples gave counts very little above background for the counter (15–20 CPM). Mean net CPM of triplicates was converted to 'mean net CPM/10^6 cells'.

The following 4 correction factors were applied:

1 Correction for the efficiency of the counter

Table I. Composition of standard cultures

Group	Cell suspension ml	Autologous plasma ml	PHA ml	^{14}C-leucine solution ml	Final ^{14}C-leucine concentration µCi/ml
Plasma free	0.5	0	0	0.5	0.5
Plasma free + PHA	0.5	0	0.06	0.5	0.5
25% plasma	0.5	0.25	0	0.25	0.25
25% plasma + PHA	0.5	0.25	0.06	0.25	0.25

L-Leucine ^{14}C(U) specific activity 150 mCi/m-mole Radiochemical Centre Amersham (England)

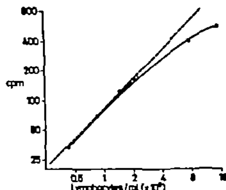


Fig 1 Effect of increasing population density in 25% plasma medium. Simultaneous triplicate 1 ml cultures of lymphocytes from the same subject were set up with increasing numbers of lymphocytes. Protein synthesis was measured after incubation for 24 h (cpm counts per minute).

2. Correction for the ^{14}C-leucine concentration to   standard ^{14}C concentration of 0.5 μCi/ml. It was shown that the CPM in newly synthesized protein were directly proportional to the concentration of radioactive substrate.

3. Correction for the amount of carrier protein to correspond to the amount (15.5 mg) in 0.25 ml of   standard pooled serum, by reference to   standard curve.

4. Correction for population density in plasma-containing cultures to give the theoretical incorporation in   culture containing 1×10^6 cells/ml, using the graph shown in figure 1 (No such correction was necessary for plasma-free cultures where the incorporation was directly proportional to the number of lymphocytes per ml up to   density of 16×10^6 lymphocytes per ml).

Results

1 Protein synthesis in plasma free medium

Mean total synthesis. The basal (unstimulated) synthesis by normal lymphocytes in plasma-free medium was measured in 28 healthy adults. The mean incorporation was not significantly different from the normal in all clinical groups CLL, lymphoma, lymphosarcoma, macroglobulinaemia, multiple myeloma and infectious mononucleosis (table II)

PHA stimulation of protein synthesis by normal lymphocytes. The stimulation by PHA is expressed as a ratio incorporation with PHA/incorporation without PHA. The effect of PHA in increasing protein synthesis by normal lymphocytes in plasma-free medium was highly significant ($P < 0.001$) The values for PHA stimulation under these culture conditions ranged from 0 7 to 3.5 with a mean of 1.87 (table II)

ing autologous plasma. Also in order to use radioactive substrate as economically as possible, the incorporation of ^{14}C-leucine into all of the soluble proteins was measured rather than into the portion which was precipitated by sodium sulphate solution. The fraction of the radioactive proteins in the globulin fraction is quite variable in normal subjects and is usually substantially less than half of the total. The results in the present study are therefore not directly comparable with the previous measurements.

These studies have provided further evidence that circulating lymphocytes may have a reduced capacity to synthesize protein in diseases involving lymphoid tissues.

Measurements of protein synthesis in plasma-free medium alone showed low activity by lymphocytes from all of the disease categories studied except multiple myeloma. However the mean total synthesis in any disease group was not significantly lower than the control mean, nor was the difference significant when the data from all categories was pooled.

The addition of 25% of autologous plasma to the medium significantly enhanced protein synthesis by normal lymphocytes. This effect is probably due to better nutrition, as unidentified substances in plasma are necessary for optimal growth of other mammalian cells *in vitro* [7]. AMBROSE [1] found hydrocortisone to be the factor in serum which was necessary for maximal antibody synthesis by rabbit lymph-node fragments *in vitro* but hydrocortisone has been shown to inhibit protein synthesis by human peripheral lymphocytes [16].

No evidence was found of inhibitory substances in the plasma of subjects with CLL, lymphoma and lymphosarcoma. The mean values for plasma stimulation of protein synthesis by lymphocytes in these conditions were actually higher than the normal mean although they did not reach the level of statistical significance. These findings are of interest in view of the suggestion of VON HALLAUER [19] that CLL serum contains substances which stimulate cell growth.

Although the mode of action of PHA and the significance of the response to it are not yet known, it is a useful substance to bring out differences in lymphocytes from different sources. When the adjuvant effects of plasma and PHA were both utilized it was possible to show significant deficiencies in the capacity of lymphocytes to synthesize protein. Significantly low activity was seen in lymphocytes from subjects with lymphoma, lymphosarcoma, macroglobulinaemia and infectious mononucleosis. Lymphocytes from patients with CLL showed

the same trend and when only the untreated cases were considered the mean incorporation was significantly lower than the normal.

The low total protein synthesis by the circulating lymphocytes in lymphoma, lymphosarcoma and macroglobulinaemia seems to be related to the poor immunological function reported in these so-called lymphoproliferative disorders [3 4 10 17]

Significantly low values for PHA stimulation in medium containing plasma were found in 2 cases of lymphosarcoma. In chronic lymphatic leukaemia one estimation was above the normal range and 3 were below The degree of stimulation by PHA was found to be significantly low in infectious mononucleosis, omitting one case in which the diagnosis was doubtful The low stimulation in infectious mononucleosis agrees well with the lack of increase of RNA synthesis in response to PHA [13] RUBIN also noted that unstimulated lymphocytes in infectious mononucleosis synthesized RNA at 10 times the rate of normal blood lymphocytes under the same conditions. This finding is not compatible with the results in our series where incorporation of C-leucine into protein was not elevated. The reason for this discrepancy is not readily apparent. It may be due to a difference in the stage of the disease between the two series more than 60% of the lymphocytes in RUBIN's cases were morphologically atypical whereas a much lower proportion of such cells was found in the blood of the subjects of this study

The cause and significance of these abnormalities of protein synthesis are not understood. Further qualitative characterization of the deficiencies is necessary It is also necessary to consider the population dynamics of the disorders, for the lymphocytes are a heterogeneous population, at least with respect to immunoglobulin synthesis [18] and each immunologically competent cell normally synthesizes antibodies of only one or two specificities [11 2] The changes in the total protein synthesis may reflect changes in the proportions of various types of cell, o changes affecting each cell of the whole population. The derangement could conceivably be initiated at the level of formation of stem cells, in the thymus, or in the peripheral lymphoid tissues. It seems likely that the disturbance in infectious mononucleosis is self limited, occurring in peripheral lymphoid tissues The disturbance may be at a similar level in lymphosarcoma, and it has been argued that the abnormalities of immunoglobulin synthesis in CLL may also be explained by a failure of terminal immunologic determination at this level [14]

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Summary

Measurements were made of protein synthesis *in vitro* by lymphocytes from normal subjects and from patients with chronic lymphatic leukaemia, lymphoma (unclassified) and lymphosarcoma, *macroglobulinaemia*, multiple myeloma and infectious mononucleosis. Protein synthesis was significantly stimulated by both plasma and phytohaemagglutinin in all groups. When the lymphocytes were cultured in medium containing 25 autologous plasma and PHA, significant differences between the normal and disease groups were observed. The degree of stimulation by phytohaemagglutinin in medium containing 25 plasma was significantly low in the lymphosarcoma and infectious mononucleosis groups. In interpreting these findings it is necessary to take int account the functional heterogeneity of the circulating lymphocytes.

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Über den Abbau von Zellen im Knochenmark des Menschen und der Ratte

D. Graf Keyserlingk

Der Abbau von Erythrozyten und Erythroblasten in den phagozytierenden Retikulumzellen des Knochenmarks ist bereits sorgfältig untersucht worden [2, 3, 4, 5]. Auch Kerne und Fortsätze von Megakaryozyten wurden in diesen Zellen gefunden [36, 38]. Es fehlen jedoch genaue Angaben darüber, welche Zellen ausserdem noch in den phagozytierenden Retikulumzellen abgebaut werden und wie dieser Vorgang morphologisch erfolgt. Bei der elektronenmikroskopischen Untersuchung dieser Frage zeigte sich darüber hinaus, dass der intrazelluläre Abbau von Zellen im Knochenmark bei Mensch und Ratte zu morphologisch verschiedenen Abbauprodukten führt.

Material und Methode

Menschliches Knochenmark wurde durch Sternalpunktion gewonnen. Ein Teil der Patienten litt an hämatologischen Erkrankungen, während bei einer zweiten Gruppe zunächst der Verdacht auf eine solche Erkrankung bestand, der dann über licht- und elektronenmikroskopisch nicht bestätigt wurde. Das Knochenmark der Ratte wurde vom Femur von 6 Tieren eines Wistar-Inzuchtstammes entnommen. Das Gewebe wurde sofort in einer 1% OsO_4-Lösung in Michaelis-Puffer mit Saccharosezusatz bei pH 7,4 und 4 °C für 1½ Std. fixiert. Stufenweise Entwässerung in Aceton und Einbettung in Micropal. Zum Nachweis der sauren Phosphatase erfolgte die Fixierung in 2,5% Glutaraldehyd in 0,1 m Cacodylat-Puffer pH 7,4. Weitere Behandlung nach der Vorschrift von Smith und Farquhar [30]. Das Inkubationsmedium enthielt 0,01 m Na-β-Glycerophosphat, 0,004 m $Pb(NO_3)_2$ in 0,0 m Acetatpuffer bei pH 4,9. Die Inkubationszeit betrug 1 Std. bei 37 °C. Anschließend Nachfixierung in 1% OsO_4-Lösung in Michaelis-Puffer. Entwässerung und Einbettung wie oben. Die Schnitte wurden mit einem LKB-Ultrotom hergestellt und mit Uranylacetat und Bleicitrat nachkontrastiert. Aufnahmen mit einem Siemens Elmiskop I.

Befunde

Bei der elektronenmikroskopischen Beurteilung von Phagozytosevorgängen muss die geringe Dicke der Schnitte berücksichtigt werden, um Fehldeutungen zu vermeiden. Die Ausläufer der phagozytierenden Retikulumzellen des Knochenmarks reichen oft weit zwischen die benachbarten Zellen. Um eine extrazellulär gelegene Zelle nicht fälschlicherweise als Phagosom anzusehen, muss deshalb eine eingeschlossene Zelle nicht nur vollständig von einer Membran der phagozytierenden Zelle umgeben sein, sondern auch Veränderungen in ihrer Feinstruktur, die für einen Abbau sprechen, erkennen lassen. Nur bei der Enzymreaktion unterscheidet sich diese Einschlussmembran schon durch ihren Niederschlag von der äusseren Zellmembran. In den phagozytierenden Retikulumzellen des Menschen und der Ratte konnten wir Zellen der roten Reihe, isolierte Normoblastenkerne, neutrophile Granulozyten (Abb. 1a), Lymphozyten, Plasmazellen (Abb. 1b) und Thrombozyten nachweisen. Da der Abbau hämoglobinhaltiger Zellen bereits mehrfach untersucht wurde [31], braucht hier nicht näher darauf eingegangen zu werden. Das markanteste Zeichen dieses Abbaus ist das Auftreten von Einschlusskörpern mit Ferritin-Granula [24] (Abb. 2). Ferritinhaltige Einschlüsse sind auch in unserem Material häufig zu finden.

Alle Einschlusskörper enthalten das Reaktionsprodukt (Bleiphosphat) der sauren Phosphatase. Wenn eine phagozytierte Zelle noch keine deutlich ausgeprägten Zeichen des Abbaus aufweist, so liegt der Bleiphosphat-Niederschlag der umgebenden Membran innen an (Abb. 2). Die äussere Zellmembran der phagozytierenden Retikulumzelle ist stets frei von Niederschlägen. Wenn deutliche Abbauerscheinungen in der eingeschlossenen Zelle erkenntlich sind, liegt das Reaktionsprodukt der sauren Phosphatase auch innerhalb des Phagosoms. Kleine Einschlüsse können vollständig von dem Niederschlag ausgefüllt sein.

Die Einschlussmembran kann der phagozytierten Zelle dicht anliegen (Abb. 1b) oder einen mehr oder weniger breiten Spaltraum zum Phagosom freilassen (Abb. 1a, 3). Dieser Spaltraum erscheint weitgehend elektronenoptisch leer (Abb. 3) oder mit einem amorphen Material geringer Elektronendichte ausgefüllt.

Im Kern der phagozytierten Zelle treten Veränderungen auf, bevor andere Abbauerscheinungen deutlich werden. Das Nukleoplasma verliert sein typisches Aussehen und wird pyknotisch (Abb. 1a)

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Investigations on the Fibrinolytic System in Liver Cirrhosis[1]

G. Tytgat[2], D. Collen[3], R. De Vreker and M. Verstraete

For nearly half a century various observers have reported increased fibrinolytic activity in the blood of patients with liver cirrhosis. However, the fibrinolytic activity was frequently measured by the autodigestion time of clots of total or diluted blood or plasma of the patient. This decreased lysis time, often due to low amounts of lysable fibrin substrate, should be interpreted as 'pseudofibrinolytic activity' as long as the lytic activity cannot be measured on a standard substrate not variable from patient to patient.

Nevertheless in some well documented cases of cirrhosis, true increased plasma fibrinolytic activity has been demonstrated. This enhanced blood proteolytic activity may be a cause of spontaneous bleeding or may encourage bleeding from esophageal varices or surgical wounds [5, 8, 11]. Particularly patients subjected to portocaval shunt operations have an increased tendency to operative and postoperative bleeding [9].

According to Fletcher *et al.* [7] the increased fibrinolytic activity in the cirrhotic patients may be due to a failure of hepatic clearance mechanisms for plasminogen activator and not to deficient plasma fibrinolytic inhibitors. This latter statement is in contradistinction with the studies of several authors [3, 11, 12, 17] indicating that the fibrinolysis in cirrhosis may also be related to decreased serum plasmin inhibitors.

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[2] Aspirant van het Nationaal Fonds voor Wetenschappelijk Onderzoek.

[3] Navorsingsstagiair van het Nationaal Fonds voor Wetenschappelijk Onderzoek.

This discordance prompted us to study both hypotheses in a group of 25 cirrhotic patients in different stages of evolution of the disease. The present investigation involved the assay of various fibrinolytic parameters before and after activation of the fibrinolytic system by nicotinic acid.

Materials and Methods

Reagents. Saline (0.07 M)-phosphate (0.06 M) buffer pH 7.5 (S.P. buffer) [10]. – Glycerol-phosphate buffer containing equal volumes of glycerol and S.P. buffer. – Fibrinogen Bovis (Poviet, Amsterdam) lot 57 (protein 78.2%; clottable fibrinogen 62.5%, NaCl 14.9%) dissolved freshly before use in a concentration of 25 mg/7 ml S.P. buffer. – Thrombin (Topostasine Roche) 20 NIH units per ml S.P. buffer stored at –20°C. – Plasmin: chloroform activated bovine fibrinolysin (Parke, Davis and Co.) 1,300 Loomis units/vial, diluted in 650 ml glycerol-phosphate buffer and stored at –20°C, is diluted adequately with the same solvent before use so that 0.10 ml plasmin lyses the standard clot (formed by mixing 0.35 ml S.P. buffer, 0.25 ml fibrinogen solution and 0.10 ml thrombin) in a 9–11 minute range. This plasmin working solution is kept on ice during the whole procedure and discarded when the lysis time of the standard clot exceeds 11 min.

Fibrinolytic activity was measured in the plasma and in the euglobulin fraction, on unheated bovine fibrin plates, prepared according to Astrup and Müllertz [1]. For statistical evaluation the individual values of lysed surface were converted into fibrinolytic activity by reading on a linear reference curve, constructed from plots of log lysed surface on bovine fibrin plate versus log sample concentration. This reference curve was obtained from a pool of fibrinolytic active euglobulin precipitates, reconstituted with veronal buffer into a number of 'activator' levels. Similar curves for individual cirrhotic precipitates showed slopes comparable to that of the reference curve.

Antiplasmin assay (according to Johnson [10] with slight modifications) 0.1 ml of serum dilutions (1/25, 1/50 and 1/100) in S.P. buffer are transferred to a testtube at room temperature. 0.15 ml of the plasmin working solution is added. The tubes are mixed gently and kept at room temperature for 10 min.

Consecutively are added: S.P. buffer 0.40 ml, fibrinogen 0.25 ml, thrombin 0.10 ml. Immediately after adding thrombin, a time reading device is started, the reagents are mixed by mechanical swirling for about 10 sec and the tube is transferred to a water bath at 37°C. Along with each series of serum dilutions a reference clot is made containing 0.10 ml plasmin dilution and buffer up to 0.65 ml. Fibrinolytic endpoints, indicated by the rising of all air bubbles into the upper half of the test mixture, are read visually or in the lysis recorder [4]. All determinations are performed at least in duplicate. The antiplasmin content of the serum is calculated from a plot on log-log paper, the lysis times being plotted against the reciprocal of the final serum concentrations. A line is drawn joining the two dots with lysis times adjacent to the reference time. On this line the serum dilution, producing a lysis time equal to the reference time is looked for. As 0.10 ml plasmin dilution contains per definition 1 plasmin unit, the reciprocal of the serum concentration which prolongs the lysis time of 0.15 ml plasmin to the time of 0.10 ml plasmin (reference time) inhibits 0.5 plasmin units. Divided by two, this reciprocal of the serum concentration gives conventionally the antiplasmin content of the sample.

Experimental groups. Nicotinic acid was administered in two series of 25 subjects. The cirrhotic group consisted of 25 hospitalized patients, in whom the diagnosis of hepatic cirrhosis, in different stages of evolution was documented by physical examination, laboratory findings, laparoscopy and proved in all of them by liver biopsy; none of the patients was taking corticosteroids, but some were under intermittent diuretic treatment. The

control group consisted of 25 laboratory personal and hospitalized psychosomatic patients without any known physical or biochemical abnormality. Mean age (controls 48 years; cirrhotics 50 years), mean body weight (controls 63 kg; cirrhotics 66 kg), mean cholesterol value (controls 238 mg%; cirrhotics 208 mg%), mean hemoglobin value (controls 14 g%; cirrhotics 13.2 g%) and sex ratio (3 males, 1 female) were comparable. Nicotinic acid (100 mg) was rapidly injected i.v. to the recumbent person in the mid-afternoon. Under these conditions, diurnal changes were assumed to operate uniformly [6].

Venous blood was sampled just before and 15, 30 and 60 min after the i.v. administration. The tourniquet was applied during about 15 sec before puncture of an antecubital vein. The blood was collected directly in glass tubes for preparation of serum and in precooled siliconized tubes, containing potassium oxalate (H_2O) 2.5% (1 part for 9 parts of blood) for fibrinolytic determinations. Serum was pipetted off after clotting of the whole blood for 2 h at 37°C. After centrifugation at 4°C for 10 min and 4,000 *g* the plasma and the serum were stored in polystyrene tubes at –20°C until used. Serum from 20 healthy donors was obtained in a similar manner and mixed in equal proportions for constituting a serum pool, used for preliminary investigation of the reproducibility of the antiplasmin assay.

Results

Determination of *in vivo* Plasminogen Activator Clearance

The fibrinolytic activity in the euglobulin fraction, measured on unheated bovine fibrin plates, will be considered according to SHERRY *et al.* as blood plasminogen activator activity [20]. Figure 1 shows the individual values of euglobulin fibrinolytic activity before (sample 0) and 15 min (sample 1), 30 min (sample 2) and 60 min (sample 3) after nicotinic acid administration.

As there is no linear correlation between lysed surface and activator

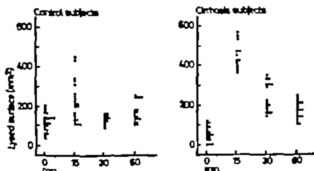


Fig. 1. Euglobulin fibrinolytic activity measured on bovine fibrin plates before and 15, 30 and 60 min after intravenous injection of 100 mg nicotinic acid, in control subjects and cirrhotic patients.

content, it is necessary for statistical analysis to compute these lysed surfaces shown in figure 1 into activator concentration by reading from a linear reference curve (plotting log mm² lysed surface versus log activator activity) After calculation of the mean activator activity in the different samples of both groups, these mean data are expressed in percentage of the mean activator activity in the control group before nicotinic acid injection. These calculated mean values are for blood samples 0 1 2, and 3 respectively 100, 206 152 and 137% for control subjects and 66, 405 274 and 154% for cirrhotic patients. The lower mean value found in the cirrhotic patients, before nicotinic acid injection is not statistically different from the mean control value ($0.05 < P < 0.10$) The increase in euglobulin fibrinolytic activity from sample 0 to sample 1 is statistically significant for both series ($P < 0.001$) the mean increase being higher for the cirrhotic patients in comparison to the control subjects ($P < 0.001$) After the 15 minute sample, the euglobulin lytic activity declined progressively to base-line values, more rapidly for the control subjects than for the cirrhotic patients.

As the *in vivo* disappearance rate of plasminogen activator is an exponential function of activator concentration with respect to time [7] the 50% *in vivo* decay time of euglobulin fibrinolytic activity can be calculated from the formula $\log Y = \log A + bx$ and $T\,1/2 = \log 2/b$. The calculated mean *in vivo* 50% disappearance time of activator is 13.8 min for those control subjects, who clearly responded to nicotinic acid injection and 24.5 min for the total group of 25 cirrhotic patients ($0.002 < P < 0.01$) The 50% *in vivo* activator decay is 26.2 min for the 10 cirrhotic patients exhibiting the most serious coagulation abnormalities and clinical signs of severe hepatic decompensation. These findings indicate that the *in vivo* disappearance of plasminogen activator in the cirrhotic patients is significantly longer compared to that of the control subjects.

Antiplasmin Determination

The reproducibility of the antiplasmin assay has been preliminarily investigated The mean of normal pool values, obtained over several weeks, is 360 antiplasmin units per ml serum, with a standard deviation of 25 units. Individual normal values vary from 210–510 units per ml, $\bar{X} = 344$ $s = 94$ and $n = 20$

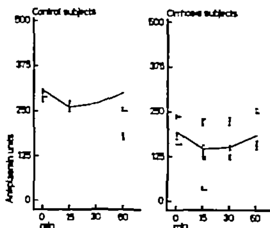


Fig. 2. Antiplasmin activity before and 15, 30 and 60 min after intravenous injection of 100 mg nicotinic acid in control subjects and cirrhotic patients.

The serum antiplasmin activity has been assayed before and after nicotinic acid injection in both experimental groups. The individual experimental results are summarized in figure 2, the mean values being for blood sample 0 1 2 and 3 respectively 311 263 271 and 296 units for the control persons and 192, 145 153 and 169 units for the cirrhotic patients. The mean antiplasmin content before fibrinolytic activation in the cirrhotic patients is significantly lower than in the control group ($P < 0.001$) Activation of the fibrinolytic system by nicotinic acid is associated in the 15 min blood sample with a decrease in serum anti plasmin content for both series, which is highly significant ($P < 0.001$) when evaluated with the method of paired comparison. The decrease in serum antiplasmin is maximal at the moment of maximal increase of euglobulin fibrinolytic activity Subsequently the antiplasmin capac ity rises steadily towards initial values, in association with a progressive decrease in euglobulin fibrinolytic activity

In order to evaluate the importance of the decreased antiplasmin level in liver cirrhosis, a comparison was made between the fibrinolytic activity on fibrin plate in total cirrhotic plasma samples versus control plasma samples both with an identical fibrinolytic activity in the corresponding euglobulin precipitates. Indeed, the difference between plasma and euglobulin lytic activity is mainly based upon the elimina tion of the bulk of inhibitors (the still hypothetical anti-activator and the antiplasmins) in the euglobulin fraction. After plotting the various

plasma fibrinolytic activities versus the corresponding euglobulin fibrinolytic activities for both series, the correlation coefficient "b" between the plasma and euglobulin lytic activity measured on bovine fibrin plate, was calculated as 0 650 for cirrhotic patients and 0.268 for control subjects. The corresponding difference in slope between both regression lines is statistically significant ($0.02 < P < 0.05$) confirming the importance of the decrease in fibrinolytic inhibitors in explaining the susceptibility of the cirrhotic patients for induction of fibrinolytic activity in total plasma.

Discussion

Various observers have reported increased fibrinolytic activity in liver cirrhosis, measured by the autodigestion time of clots of total or diluted blood or plasma of the patient. In our experiments, the lytic activity before nicotinic acid injection was measured on a standard lysable fibrin substrate, not variable from patient to patient, and no evidence for enhanced plasminogen activator activity in the euglobulin fraction of 25 cirrhotic plasma samples was obtained (fig 1) Nevertheless, patients with liver cirrhosis are prone to develop increased fibrinolytic activity in certain circumstances [8, 9 19] Many authors relate the increased fibrinolytic activity to the release of an increased amount of plasminogen activator in the cirrhotic plasma.

Our data confirm the known susceptibility of the patients with liver cirrhosis to develop increased fibrinolytic activity after stimulation of the fibrinolytic system (fig 1) According to FLETCHER *et al.* [7] a major factor in the abnormal fibrinolytic response to nicotinic acid, is a failure of hepatic clearance mechanisms for plasminogen activator in liver cirrhosis. Calculating the activator concentration by assessment of the euglobulin lysis time, these authors find a mean *in vivo* 50% plasma activator clearance rate of 13 ± 5 min in control persons versus 53 ± 19 min in cirrhotic patients.

In our experiments blood plasminogen activator precipitated almost quantitatively in the euglobulin fraction, is measured on bovine fibrin plates. From our data the calculated mean *in vivo* 50% disappearance time of activator activity is 13 8 min for the control subjects, who clearly responded to nicotinic acid injection, and 24.5 min for the total group of 25 cirrhotic patients ($0.002 < P < 0.01$) Notwithstanding the methods in both studies differ the *in vivo* disappearance of plasminogen activator in the two control series is

strikingly similar However the prolongation of the *in vivo* decay of plasminogen activator in cirrhotic patients in comparison with control subjects is less pronounced in our study As differences in patient material (our patients being scattered over a wider range of liver insufficiency) could provide a possible explanation for this discrepancy we calculated the *in vivo* plasminogen decay in the 10 cirrhotic patients with the most seriously disturbed coagulation set and clinical signs of severe liver failure, but the 50% plasminogen decay time of 26.2 min in this group was still the half of the value found by FLETCHER [7] The significantly longer *in vivo* disappearance time of plasminogen activator *in cirrhotic patients, compared to control subjects*, is according to FLETCHER [7] ascribed to insufficient hepatic clearance mechanisms of activator by the diseased liver However it has to be kept in mind that other still hypothetical mechanisms may be involved in explaining the differences in activator disappearance time after nicotinic acid injection, such as differences in possible renal clearing of activator activity differences in continuous activator release during the declining part of the disappearance curve, differences in nicotinic acid metabolism, and differences in level and rate of release of anti blood activator activity The availability of purified blood plasminogen activator is required for exact activator clearance studies and for further elucidation of the mentioned hypothetical mechanisms.

The role of the fibrinolytic inhibitors in the genesis of the increased fibrinolytic activity in liver cirrhosis and the enhanced susceptibility to stimuli capable of inducing fibrinolytic activity is debated. According to FLETCHER *et al.* [7] assays of plasma antiplasmin, performed with a caseinolytic technique, revealed no significant difference between the normal and cirrhotic group. Studies of other authors however [3 11 12, 17] indicate that the fibrinolytic activity in liver cirrhosis may be related to a decrease in the plasmin inhibitors in serum, this finding being in agreement with the previously stated lowered levels of alpha$_2$-globulins in cirrhosis [18] We performed a serum antiplasmin assay in 25 cirrhotic patients according to the fibrin clot digestion method as proposed by JOHNSON [10] Interference in the test by residual activator activity in the serum samples was considered as *negligible if not inexistent both for reasons of prolonged clotting* at 37° C of the blood and subsequent extensive dilution (250–1 000 times) of the serum sample. As autodestruction of plasmin occurs during the test, the error may be minimized by measuring serum inhibition after a relatively short incubation time (10 min) and by adding glycerol as a

stabilizer to the plasmin solution. Although the plasmin inhibition is not fully evolved, this short preincubation time may be reliable as the bulk of serum antiplasmin activity resides in the $alpha_2$-globulin fraction, which produces an immediate temperature independent inhibition of plasmin [13 15 16] The mean antiplasmin content (192 U/ml serum) in the cirrhotic patients is substantially lower than the corresponding value in control subjects (311 U/ml serum) this difference being highly significant ($P < 0.001$) (fig 2) The importance of the decrease in fibrinolytic inhibitors (the still hypothetical anti-activator and the antiplasmins) was evaluated by comparing the lytic activity in cirrhotic versus control plasma samples with an identical corresponding lytic activity in the euglobulin fraction where the bulk of fibrinolytic inhibitors is eliminated. Our finding of a significantly higher plasma fibrinolytic activity in cirrhotic plasma in comparison with control plasma for an identical euglobulin lytic activity supports the hypothesis that the susceptibility of the cirrhotic patients for induction of fibrinolytic activity in total plasma may be related to decrease in fibrinolytic inhibitors ($p < 0.05$) Decreased antiplasmin levels in cirrhosis are probably not only due to decreased hepatic synthesis but also to increased consumption in plasmin antiplasmin complex formation. Indeed it has been demonstrated that plasmin-antiplasmin complex may form a circulating reservoir of plasmin which is innocuous to other circulating proteins but forms a ready source of plasmin when required for the lysis of fibrin. The extent of such plasmin binding may be appreciated from the work of NANNINGA and GUEST [14] who have shown that bound plasmin is present in a hundredfold greater concentration than free plasmin at a plasmin concentration of 10^{-7} M

There is a suspicion that not only the antiplasmin content but also the level of the still hypothetical inhibitor of blood plasminogen activator is decreased in liver cirrhosis. As it has repeatedly been shown that increased fibrinolytic activity is frequently the result of the presence of an activator of plasminogen and not of plasmin an inhibitor of this activator may have important physiological implications. However it has until now been impossible to measure such an inhibitor as the activator itself has not been isolated and purified.

Summary

Patients with liver cirrhosis are prone to develop increased fibrinolytic activity after *i.v.* injection of nicotinic acid. This abnormal response is due to failure of hepatic clearing of plasminogen activator. The calculated mean in vivo 50% disappearance time of activator activity is 13.8 min for 25 control subjects and 24.5 min for 25 cirrhotic patients ($0.002 < p < 0.01$). Decreased antiplasmin levels in cirrhotics may potentiate the susceptibility of these patients to develop plasma fibrinolytic activity. This difference between the mean antiplasmin content (192 U/ml serum) in the cirrhotic patients and the corresponding value in the control subjects (311 U/ml serum) is highly significant. The decrease in fibrinolytic inhibitors is probably related with the finding of significantly higher fibrinolytic activity in total cirrhotic plasma in comparison with control plasma for an identical level of plasminogen activator activity in the corresponding euglobulin fraction of both cirrhotic and normal plasma ($0.02 < p < 0.05$).

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A Specific Inhibitor of Human Clotting Factor V

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In 1958 FERGUSON *et al.* [5] reported on a patient suffering from a heavy haemorrhagic diathesis due to the presence of an inhibitor of factor V. This inhibitor showed no species-specificity since it was found to inhibit bovine factor V as well. The therapeutic effect of ACTH suggested that this anticoagulant could have been of immunological origin although no direct proof for this assumption was offered.

We have observed a similar coagulation disorder in a 66-year-old woman. An inhibitor which was effective only against human factor V could be demonstrated in the plasma as well as in the serum of the patient.

Case Report

M.R.L. 1899, was admitted to the hospital on September 26th, 1965 because of a right-side pleurisy. Prior to hospital admission she had been treated with chloromycetin, Tanderil® and Lederkyn®. From her history it became clear that she had suffered from a specific (tbc) lung infiltration already 40 years ago.

After a few days in the hospital the patient developed a pericarditis. Tuberculostatic therapy was started. The treatment of a thrombophlebitis of the right leg which developed early in 1965, was followed by prolonged Butazolidin® therapy. The patient's condition had improved significantly by November 15th, 1965 and she was discharged from the hospital; however 6 days later the patient had to be readmitted as an emergency with haematuria which had persisted for 2 days already. On the medial side of the right thigh and on the abdomen small to medium-size suffusions were visible. The patient was afebrile. A cystoscopy gave no evidence for mucous membrane lesions.

Laboratory findings: haemoglobin 54%, haematocrit 21%, bleeding time 3½ min, clotting time 5 min, thromboplastin time determined according to Quick 55%, thrombocytes 136,000/mm³.

The patient received blood transfusions; in spite of serological compatibility she developed a temperature of 39.2° C after the fourth transfusion.

An analysis of the blood clotting system was performed on a blood sample received on November 23rd, 1965 (table I). The most striking result consists in the pronounced reduc

tion of factor V activity as measured by the one stage assay. Correspondingly the global tests of the intrinsic system (recalcification time, hirudin tolerance time, partial thromboplastin time and prothrombin consumption test) as well as thromboplastin time were all pathological. The moderately diminished factor XI activity was probably due to the low factor V content of the factor XI-substrate used.

The patient's history gave no indication of parahaemophilia; she never had bleeding episodes before although she was allergic to penicillin. Furthermore, a hereditary coagulopathy seemed excluded by the absence of bleeding disorders in the patient' family. Thus, the presence of an inhibitor of factor V appeared probable.

The patient's blood was reexamined on December 4th, 1963 (table I). Factor V activity was still markedly reduced, and hyperfibrinogenaemia had developed.

A search for auto- and iso-antibodies against red cells, for leucoagglutinins, for cell nucleus antibodies, for rheumatoid factor activity and for antibodies against immunoglobulins gave negative results.

Special Investigations

1. Substitution experiments in Quick's thromboplastin test. The following incubation mixture was used: 0.1 ml citrated plasma (patient's and normal pooled plasma mixed in different ratios), 0.1 ml thromboplastin 'Roche', 0.1 ml 0.4 M $CaCl_2$. The results of substitution experiments using the patient's plasma (M-plasma) and normal plasma are shown in figure 1.

In a second series, cross-correction experiments were performed using different incubation times of the patient' plasma with normal plasma. In these experiments the plasma was diluted 1:10 with isotonic barbital buffer (figure 2). The results obtained must be interpreted in terms of the presence of an anticoagulant inhibiting the prothrombin-conversion by the extrinsic activation mechanism. The activity of this anticoagulant appeared immediately but increased on prolonged preincubation.

2. Effect of the inhibitor on thromboplastin generation. As shown in figure 3, the thromboplastin generation test (TGT) [3] in which $BaSO_4$-absorbed patient's plasma and normal

Table I. Clotting investigations

	23-11-63	4-12-63	5-1-66	Normal range
Recalcification time, sec	450	840	115	90-180
Hirudin tolerance time, sec	570	1,200	120	~300
Partial thromboplastin time (Azolectin), sec	300	510	95	70-100
Prothrombin consumption test, %	45	55	3	0-8
Thromboplastin time, sec	55	63	12	~13
Thrombin time, sec	11	16	14	~10
Fibrinogen (Schulz), mg %	400	600	300	~300
Factor II activity, sec	19	17	18	~19
Factor V activity, %	5	5	60	70-120
Factor VII activity, %	90	–	100	70-120
Factor VIII activity, %	100	100	100	70-120
Factor IX activity, %	100	100	–	70-120
Factor X activity, %	100		100	70-120
Factor XI activity, %	22			70-120

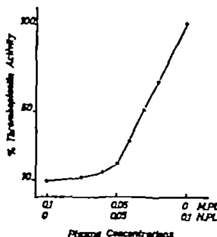


Fig 1 Substitution experiments in Quick's thromboplastin test. *M.Pl.* patient's plasma, *N.Pl.* normal plasma.

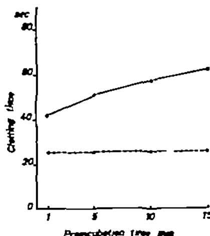


Fig.2. Cross correcting experiments with patient's plasma and normal plasma t different preincubation times. ●——● mixture of patient plasma and normal plasma; ●——● normal plasma alone.

serum were used, is also influenced by the inhibitor This experiment shows, furthermore, that the inhibitor is not or only poorly absorbed by BaSO I proved necessary to modify the standard conditions of the TGT whereby concentrated reagents ($BaSO_4$-absorbed plasma and serum) were used in order to demonstra; the activity of the inhibitor

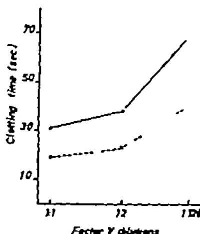


Fig 7 Activity of human and bovine factor V preparations. ●——● human factor V preparation ●------● bovine factor V preparation.

shown in figure 5. They appear to demonstrate the presence of an anticoagulant directed against factor V. These findings also confirm that the inhibitor is present in both plasma and serum, and that it is effective upon incubation.

Patient's serum and normal plasma were preincubated for different periods of time in order to establish the interdependence of the inhibitory effect and preincubation time; the one-stage factor V assay was used. The results represented in figure 6 demonstrate that the inhibitory effect increases correspondingly with increasing incubation times.

4 Inhibition experiments with factor V-preparations of bovine and human origin. Factor V was prepared according to a slightly modified version of OWREN's method [15]. The activity of the lyophilised product was tested as follows: 25 mg of factor V preparation of bovine origin and 100 mg factor V preparation of human origin, respectively, were dissolved in 1 ml barbital buffer. Factor V activity was determined in dilutions prepared from these solutions. Both preparations were tested for activity of other clotting factors: only factor V activity could be demonstrated.

For demonstration of inhibitor activity both, bovine and human factor V-preparations were adjusted to equal activity as based on their respective activity curves (figure 7). 0.5 ml factor V-preparation (human or bovine) was preincubated with 0.05 ml of the patient plasma for different lengths of time: the clotting activity was measured by determination of the thromboplastin time. As a control, the patient' plasma was substituted by factor V reagents. Results are shown in figure 8, demonstrating that the inhibitor is specifically directed against human factor V but not effective against bovine factor V. Furthermore, it appears from the shape of the curve that both preparations may have lost some of their activity during the test procedure.

5. Stability of the anticoagulant. The inhibitor showed no loss of activity after standing at +4°C or -20°C for several weeks. Incubation at 37°C for 24 h likewise caused no loss of activity. After 1 h at 60°C no decrease of activity was discernible, whereas at 70°C the activity disappeared after 30 min.

6. Chemical and physicochemical properties of the anticoagulant. The inhibitor was neither absorbable on aluminium-hydroxide nor on $BaSO_4$ [2]. It is not lipophilic since after shaking with ether it was still found in the water phase.

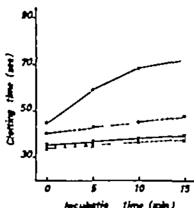


Fig. 8. Dependence of the inhibitor's activity against factor V preparations on preincubation time

●——● human factor V preparation + patient plasma
●- - -● human factor V preparation + factor V deficient plasma
×——× bovine factor V preparation + patient's plasma
×- - -× bovine factor V preparation + factor V deficient plasma

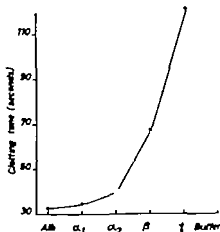


Fig. 9. Distribution of the inhibitor activity among electrophoretic fractions of the patient's serum.

In order to determine the electrophoretic mobility of the inhibitor 0.1 ml of the patient serum was applied to each of 13 paper strips. After electrophoresis, one strip was colored with amido black to localise the different fractions, and the 5 main electrophoretic fractions thus localized were cut out in the remaining 12 strips. The paper cuttings were eluted during 12 h in barbital buffer at 4 C, whereby the amount of barbital buffer added was proportional to the protein content of the different fractions. Of each eluate 0.5 ml were

mixed with 0.1 ml of normal plasma, diluted 1:10 with isotonic barbital buffer during 30 min. Factor V activity was then assayed by the one-stage test. Figure 9 shows that the inhibitory activity was contained mainly in the γ-globulin fraction.

7. Immunochemical investigations. The patient's serum was submitted to OUCHTERLONY immunodiffusion tests against human and bovine factor V-preparations, against normal pooled plasma, as well as against factor V-free plasma (factor V reagent). A weak but definite precipitation line formed between the patient's serum and human factor V preparation, whereas reaction of the patient's serum with normal plasma pool remained doubtful. No reaction was observed between the patient's serum and bovine factor V preparation, and no precipitation line was formed, as expected, between the patient's serum and factor V free plasma. Due to the weak appearance of the precipitation line it was not possible to obtain convincing photographic picture for reproduction.

Follow-up Studies

On December 18th 1965, new sample of citrated blood was tested. No alteration in the clotting defect was discernible. Factor V activity was still only 3% of normal values.

The patient general condition was unchanged. After transfusion, haematuria stopped for a few days, but then bleeding resumed. On December 20th, 1965, ACTH-therapy was started. A daily dose of 25 mg was given until January 8th, 1966. A few days after initiation of this therapy the general condition improved both objectively and subjectively haematuria stopped and the thromboplastin time showed substantial improvement amounting to 60% by December 29th, 1965. On January 5th, 1966, the coagulation analysis was resumed. With the exception of a slightly reduced factor V activity (60% of normal values) blood coagulation appeared normal (table I). On January 9th, 1966, the patient went to the bathroom where she suddenly collapsed and died. An embolic obstruction of the pulmonary artery as well as thromboses of the left femoral and iliacal veins were revealed at autopsy.

Discussion

The appearance of haemorrhagic diathesis caused by the action of inhibitors of the clotting system is well known. This phenomenon may be due to either a raised activity of physiological inhibitors (e.g. antithrombins) or the appearance of (usually acquired) circulating anticoagulants (e.g. specific inhibitors of factor VIII). Circulating anticoagulants are most frequently directed against factors VIII and IX or against tissue thromboplastin however specific inhibitors of most other clotting factors may be found as well [8].

Formation of circulating anticoagulants specifically directed against factor V appears to be very rare. In addition to the one case mentioned by FERGUSON *et al.* [5] there exists only the observation reported by HÖRDER [9, 10] in 1955. A critical evaluation of HÖRDER's case especially with regard to the genetic pattern of the haemorrhagic diathesis and to his interpretation of the experimental data leaves it very doubtful that a factor V inhibitor was present at all. We are inclined to agree with FERGUSON *et al.* who concluded that HÖRDER described a case of parahaemophilia.

The case reported in this study is very similar in its characteristics to the one described by FERGUSON. As a result of the inhibition of factor V an impairment of the prothrombin-conversion by the intrinsic as well as by the extrinsic activation mechanisms occurs: accordingly the recalcification time the hirudin tolerance time, the partial thromboplastin time, as well as the thromboplastin time are distinctly prolonged. Inhibition experiments with purified factor V preparations clearly demonstrate the factor V specificity of the inhibitor. However the inhibitor present in our case, contrary to the inhibitor of FERGUSON *et al.*, did not react with bovine factor V and therefore appears to be species-specific. Furthermore it may be concluded from inhibition experiments, that the inhibitor is exhibiting immediate as well as progressive activity. Evidence indicating that the inhibitor could be an antibody appears to be of particular interest. This concept is supported by the physicochemical properties (electrophoretic behaviour as a γ-globulin) by the effect of ACTH-therapy but mainly by the results obtained in immunochemical analyses. In fact the inhibitor behaved as a weak precipitin: it must be considered, however that most precipitating antibodies form only after prolonged and powerful antigenic stimulation.

For a long time, most authors were inclined to believe that acquired circulating anticoagulants were antibodies [8]. Inhibitors found in multi-transfused haemophiliacs are classified as isoantibodies since these patients do not possess the corresponding antigens (factor VIII or factor IX, respectively). Inhibitors observed *post partum* often can be related to an iso-immunization: in this case, one must postulate group-specific differences in the antigenic structure of the clotting factor in question. However such differences have not as yet been demonstrated.

Inhibitors detectable in cases of collagen disease (e.g. in patients with *lupus erythematodes disseminatus*) but also during the course of other diseases, such as chronic glomerulonephritis, liver cirrhosis, tuberculous lymphadenitis, sepsis lenta, lues, pemphigus vulgaris, dermatitis herpetiformis, are likewise attributed to an immunologic origin. In all these cases, an autosensitization must be assumed. Indirect support for the concept of the antibody character of circulating anticoagulants in most instances may be gained from the patient's history or from the physicochemical behaviour of the inhibitor. Direct serological evidence has been presented only in a few cases: occasionally precipitating antibodies against factor VIII or factor IX could be demonstrated

[4, 6, 11, 12, 18, 19]; in other cases the inhibitor activity could be removed with a specific anti-immunoglobulin anti-serum [2, 16, 17]. To our knowledge, an inhibitor with antibody activity directed against factor V has never been demonstrated before. In our case the antibody may be of autoimmune origin, although the question of aetiology has to remain open. Neither clinical nor serological evidence for a generalised autoimmune disease was found. The aetiological role of drugs in the induction of autoantibody formation has to be considered. As mentioned already, the patient was allergic to penicillin. STEFANINI and DAMESHEK [17] reported on a patient with purpura SCHÖNLEIN-HENOCH, who developed a circulating anticoagulant directed against factor VIII after the administration of penicillin procaine; simultaneously the clinical picture deteriorated. Dysproteinaemia of the γ-globulin type observed in this patient was traced back by the authors to the presence of large amounts of the inhibitor, although no direct proof for its antibody character was offered. An analoguous case has also been described by NUSSEY and DAWSON [13].

Acknowledgement

We are greatly indebted to Dr. H. V[illegible], Langnau, for putting blood samples as well as the case history and clinical data of his patient at our disposal.

Summary

A severe haemorrhagic diathesis in a 66-year-old woman was found to be due to a circulating anticoagulant directed against clotting factor V. The inhibitor was species-specific and active immediately. Immunochemical studies presented evidence for the antibody character of the inhibitor.

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Another Family Showing the Interaction of the Genes for Hb G and Hb S[1]

L. E. Lie Injo, A. C. Wang[2] and R. C. Burnett

The combination of haemoglobin $G\alpha$ and haemoglobin $S\beta$ is rare. Puoit *et al.* [12] described a family in which this abnormality resulted in 4 major haemoglobins in one individual and in mild anaemia. To our knowledge no other instance of Hb $G\alpha$-Hb $S\beta$ combination has so far been reported. More frequent are instances of Hb $G\alpha$ combined with Hb $C\beta$, which also lead to the presence of 4 haemoglobin components and to mild haematological symptoms. Smith and Torbert [18] described 4 haemoglobin components in cases of Hb Hopkins 2 – Hb S, and Hall-Craggs *et al.* [5] in cases of Hb S – Hb Stanleyville II. Hb Hopkins 2 and Hb Sta II are also abnormal in the α-chains. Family studies in such cases revealed that the mutants of the α- and β-chains were inherited independently of one another. The study of such exceptional families is essential to understand the mode of inheritance of haemoglobin abnormalities and to obtain a better idea of the arrangement of the genetic loci in the chromosomes. The more families that are studied, the better one can calculate the probable relative location of the genes.

We therefore believe it important to report another case of Hb $G\alpha$ combined with Hb $S\beta$. It provides more data on the clinical and haematological expression of such an interaction and on the chromo-

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[2] Postdoctoral Fellow (No. PF-407) from the American Cancer Society.

somal relationship of the genes responsible for abnormal haemoglobins in an additional family

Methods

Haematologic examinations were conducted according to standard methods. Haemoglobin electrophoresis was performed on starch gel [19] and on agar gel [15]. Alkali denaturation of haemoglobin followed the method of Singer et al. [17] with 2% of the total amount of haemoglobin taken as the upper limit of normal. The solubility test was performed by the method of Itano [9]. Haemoglobin components were quantitatively analyzed on diethylaminoethyl (DEAE) cellulose column chromatography by the method of Huisman and Dozy [7]. Polypeptide chains of haemoglobin were studied on starch gel electrophoresis in an urea-barbital system at pH 8.0 [4]. Fingerprinting of peptides was performed by Baglioni's [2] modification of Ingram's method [8].

Isolation of peptides. Peptides for amino acid analysis were isolated on Whatmann 3MM filter paper by one-dimensional electrophoresis followed by descending chromatography as described by Ingram [8]. Additional purification was achieved by descending chromatography in butanol, acetic acid, and water solvent (4 1 5 upper layer 24 h). After each step of separation, guide strips were cut from both sides of the paper and dipped in ninhydrin to locate the position of the band containing the peptide to be isolated. This region was then eluted with solvent of ethanol, acetic acid and water (50 0.15 49.85) pH 3.8, and the eluate was evaporated to dryness in desiccator.

Amino acid analysis. The isolated peptides were hydrolyzed in 6 N HCL under reduced pressure at 110°C for 20 h, and the amino acids were analyzed on Spinco automatic amino acid analyzer (Model 120 C).

Case Report

H. E., the propositus, was 45-year-old Negro man who complained of prolonged tiredness. Upon examination he was somewhat pale but nonicteric and otherwise physically normal, without enlarged spleen or liver. Repeated blood examinations revealed mild anaemia with slight microcytosis. Haematological findings, when the patient was examined again on September 21 1967 after repeated iron therapy were as follows: Hb 12.5 g per 100 ml, RBC 5.40 million/mm³, PVC 39.0%, MCV 72.2 μm³, MCH 22.8 pg, MCHC 31.5%, reticulocytes 2.1%. WBC and platelet counts normal. The peripheral blood smear showed slight anisocytosis and poikilocytosis with few target cells. Sickling of the erythrocytes took place, although not readily when they were deprived of oxygen. Alkali-resistant haemoglobin was 0.5% of the total amount of haemoglobin. Acid elution for Hb F was negative. Serum bilirubin was not increased. Haemoglobin electrophoresis revealed 4 components (1) Hb A, (2) haemoglobin component with the mobility of Hb S, (3) component with the mobility of Hb A_2, and (4) slower haemoglobin component more cathodic than Hb A_2 and resembling Hb B_2 (fig. 1).

Examination of the patient's wife 2 daughters, and 3 sons disclosed the following: S. E., the 32-year-old Negro wife, was physically and haematologically normal. Her haemoglobin electrophoretic pattern was also normal, she showed no increase of fetal haemoglobin and her Hb A_2 level was not elevated. The 2 daughters, Qd and Qt, 15 and 14 years old, were physically normal. Their peripheral blood showed slight microcytosis, anisocytosis, and poikilocytosis and contained few target cells. Results of the sickling test were positive, but sickling did not take place readily. The pattern of haemoglobin electrophoresis in these 2 daughters was identical to the father's, again consisting of 4 haemoglobin components

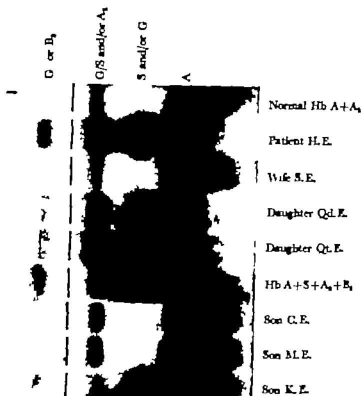


Fig. 1. Starch gel electrophoresis in tris-EDTA-boric acid buffer pH 8.6 of the haemolysates of patient and family.

(fig. 1). Hb F was not increased. All 3 sons were physically normal. C. E., the eldest, 17 years old, was haematologically normal and had normal haemoglobin pattern on electrophoresis, normal Hb A_2 level, and no increase of Hb F. K.E., the 11-year-old second son had haemoglobin pattern like that of the father and two sisters. It consisted of 4 haemoglobin components, but their quantitative distribution differed (see 'Further Haemoglobin Studies') and his erythrocytes did not sickle. M. E., the 10-year-old youngest son had normal electrophoretic haemoglobin pattern without increase of Hb F or Hb A_2. The haematological findings in all members of the family are listed in table I. After prolonged oral iron therapy haematological studies were repeated in all members of the family but they were found to be essentially unchanged.

Further Haemoglobin Studies

Electrophoresis. Haemoglobin findings on starch gel electrophoresis have already been presented (Case Report). On agar gel electrophoresis, the haemolysate of the patient and the 2 daughters, whose red blood cells

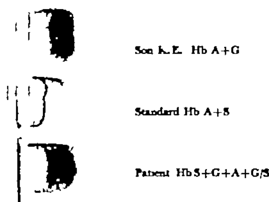


Fig. 2. Agar gel electrophoresis in citric acid-sodium citrate buffer pH 6.2 of patient haemolysate compared with that of his son K. E. and standard Hb A + S. Note that there is no separation in the haemolysate of son K. E.

showed sickling, revealed two clearly separated components, the slower-moving one having the same mobility as a standard Hb S (fig 2). This slower component was smaller than the component with the mobility of Hb A on agar gel electrophoresis, contrary to the finding on starch gel electrophoresis. The wife and 3 sons, including K. E., who showed a component like Hb S on starch gel electrophoresis but whose erythrocytes did not sickle, each had just one band like normal Hb A, indicating that in K. E. the slow moving Hb S like component, on starch gel electrophoresis was not Hb S. This explains the inability of his erythrocytes to sickle.

Column chromatography. Quantitative analysis of haemoglobin components of family members was made by column chromatography. These components in the propositus were Hb A 39.5%, the component with the mobility of Hb S 43.9%, the one with the mobility of Hb A_2 14.6% and the slowest component 2%. Percentages for the same components in the eldest daughter were 37.0, 46.8, 14.8 and 1.4% respectively. Insufficient blood was obtained from the second daughter to perform quantitative analysis on column chromatography. Data for K. E., with a component like Hb S on starch gel electrophoresis, were Hb A 70%, the component like Hb S 26.9%, Hb A_2 2 and the slowest component 1.1%. To further demonstrate that the component like Hb S in this son was not Hb S we separated and purified it by column chromatography. A solubility test showed that this component, unlike solutions containing Hb S was fully

Table I Haematological data in patient and family members

	Sex	Age years	Hb g/100 ml	RBC 10^6/mm^3	Hct %	MCV μ^3	MCH pg	MCHC %	Reticulocytes %	Hb A %	Hb S or Hb G %	Hb G/S[1] and Hb A_2 %	HbA$_2$ %	HbG$_2$ %	Hb alk. resistant %
H. E., propositus	M	43	11.8	5.06	38.0	75.1	23.3	30.5	2.1	39.5	43.9	14.6		2.0	0.5
S. E., wife	F	32	13.6	4.80	45.0	89.6	28.3	31.6	1.4	87.6	0	–	2.4	0	1.0
O. E., son	M	17	12.7	4.95	41.0	82.8	25.7	31.0	0.5	97.5	0		2.5	0	1.5
Qd. E., daughter	F	15	12.2	5.25	36.0	68.6	23.2	33.9	1.6	37.0	46.8	14.8	–	1.4	0.8
Qt. E., daughter	F	14	12.6	5.70	38.5	67.5	22.1	32.7	1.0	n.e.	n.e.	n.e.	n.e.	n.e.	0.9
K. E., son	M	11	11.9	5.04	36.0	71.2	23.6	33.1	0.8	70.0	26.9	–	2.0	1.1	1.8
M. E. son	M	10	11.9	4.90	38.0	77.8	24.3	31.3	0.6	97.5	0		2.5	0	0.6

[1] Hb A_2 cannot be estimated in the presence of Hb G/S.
n.e. Not examined.

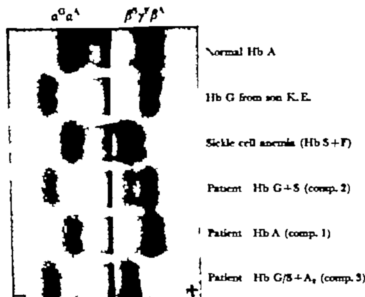


Fig. 3. Starch gel electrophoresis in urea-veronal buffer pH 8.0 of the polypeptide chains of purified haemoglobin components.

soluble in the standard phosphate buffer solution. The haemolysates of the mother and the 2 other sons separated only into Hb A and Hb A_2, the Hb A_2 level being normal (2.4% in the mother and 2.5% in the eldest and youngest sons).

Polypeptide chain studies. The different components were separated by column chromatography, concentrated, dialyzed, and their purity was determined on starch gel electrophoresis. The study of globins from the purified haemoglobin samples on starch gel electrophoresis in a urea veronal buffer at pH 8.0 revealed that the haemoglobin with the mobility of Hb A in the propositus and his 2 daughters indeed had normal α- and β-chains. The component with the mobility of Hb S however had 4 polypeptide chains: normal α- and β-chains and abnormal α- and β-chains (fig. 3). The abnormal β-chains resembled those of Hb S. These findings indicate that the component with the mobility of Hb S actually consisted of 2 different haemoglobins: (1) Hb S (whose presence explained the positive sickling) and (2) a haemoglobin whose mobility was similar to that of Hb S on starch gel electrophoresis at pH 8.6 but which had abnormal α-chains. The patient's haemoglobin component with the mobility of Hb A_2 showed a similar abnormal α-chain and an abnormal β-chain resembling that

Table II Amino acid composition of the 9a and 9b peptides of the second son K.E.

Amino acid	Peptide 9 Molar ratio Present work	Peptide 9 Molar ratio Literature value for Hb G	Number[1] of residues reported for Hb G	Peptide 9b (= 5b) Molar ratio Present work	Peptide 9b (= 5b) Molar ratio Literature value for Hb G	No. of residues reported for Hb G
Lysine	1.82	1.91	2	0.99	0.81	1
Histidine				3.04	2.28	3
Aspartic acid	1.19	1.09	1	4.39	4.01	4
Threonine	0.94	0.87	1			
Serine				1.76	1.83	2
Proline				0.90	1.04	1
Alanine	1.80	2.13	2	5.13	4.92	5
Valine	1.14	0.96	1	2.09	2.03	2
Methionine				0.16	(1)	1
Leucine	1.00	1.00	1	3.00	3.00	3

[1] Baglioni and Ingram [3].

The greatest portion of the methionine in our analysis was oxydized to methionine sulfoxyde, which appears on the chromatogram as peak before the aspartic acid peak [11].

Discussion

From the clinical and haematological findings for the patient and his two daughters, in all of whom the abnormal genes for Hb G and Hb S were present simultaneously, we concluded that interaction between the 2 abnormal genes does not lead to significant impairment of health. However, mild anaemia may be present, and their peripheral blood showed slight anisocytosis and poikilocytosis and a few target cells. In the case of simultaneous occurrence of Hb G and Hb S described by Pugh *et al.* [12] the patient, a child, had severe anaemia and clinical symptoms. However, the condition in that case was complicated by homozygosity for the Hb S gene itself leading to severe anaemia. The child's mother, on the other hand, was doubly heterozygous for Hb G and Hb S and had only mild anaemia and slight changes in the peripheral blood.

Biochemically, our patient and his two daughters, all with the same abnormal haemoglobin pattern, again provide evidence that normal and abnormal α-chains and normal and abnormal β-chains are produced independently, as reported by other investigators. Identical peptide chains form symmetrical dimers, which combine at random and

lead to the formation of 4 major haemoglobins. As in the case of PUGH *et al.* [19] 2 haemoglobins besides Hb G and Hb S are formed, Hb A with normal α and β-chains and Hb G/S with abnormal α- and β-chains. Figure 3 shows that in our patient's component 2, which consists of Hb G and Hb S the spots indicating α^G and β^A are more pronounced than the spots indicating α^A and β^S which means that the amount of Hb G is higher than Hb S This is in agreement with the observations of PUGH *et al* in the only other known family with Hb G and Hb S Using a different quantitative technique they showed in their patient's mother who carried the genes for Hb G as well as for Hb S that the amount of Hb G was higher than the amount of Hb S They postulated that the presence of some type of non-allelic genetic regulator might have impaired the normal α-chain production. The decreased availability of α_2^A subunits would then result in lower proportion of Hb A and Hb S Whether this is always the case in Hb G and Hb S combinations cannot be said since the family of PUGH *et al.* and the one described in this paper are the only ones available for study

Genetically the family we have described is of interest because the propositus, who had two abnormal genes, was married to a normal woman. Since 2 of his children inherited both his abnormal genes, one child inherited only one abnormal gene, and 2 inherited no abnormal haemoglobin, it can be concluded that the genes are not alleles. Hence, the 2 abnormal genes are either located in 2 different chromosomes that were inherited independently or are linked in one chromosome but one son received only one abnormal gene due to crossing over Unfortunately descriptions of such critical matings have been too few to permit a more accurate evaluation of the chromosomal relationship of the genes. In the family reported by PUGH *et al.*, in which the genes for Hb G and Hb S were segregating, the observed inheritance of Hb G and Hb S was complicated because the husband was a Hb S trait carrier and therefore not normal. One child from this mating was heterozygous for Hb G and homozygous for Hb S, another was normal, and the other children were not available for study

As already mentioned, the segregation of the genes for Hb G and Hb Cβ has been described more often than the combination of Hb Gα and Hb Sβ. Since the genes for Hb C and Hb S are alleles, the mode of inheritance for Hb C also applies for Hb S and information regarding the chromosomal relationship between the Hb Sβ and Hb Gα genes can also be derived from families in which Hb C and Hb G are

segregating. Unfortunately, again in the publications about such combinations, critical matings for genetic study were rarely reported. In the family described by RAPER *et al.* [13] a man doubly heterozygous for Hb G and Hb C was married to a normal woman and they produced one doubly heterozygous child and one Hb C trait carrier. Matings less suitable for genetic information have been reported by others. In the family described by WEATHERALL *et al.* [20] a man with the Hb C trait married a woman heterozygous for Hb G. Of the 5 offspring of this mating who were examined, one was normal, one heterozygous for Hb G and 3 were doubly heterozygous for Hb G and Hb C. In the family reported by RIEDER and NAUGHTON [14] a man homozygous for Hb C married a woman heterozygous for Hb G and they produced one child who was doubly heterozygous for Hb G and Hb C. In this case the Hb G was Hb G Baltimore instead of Hb G Philadelphia. In the family with Hb G and Hb C described by ATWATER *et al.* [1] the husbands of the women reported were not examined. Another combination of an α-chain abnormality with a β-chain abnormality was reported by HALL-CRAGGS *et al.* [5] in a family in which the genes for Hb S and for Hb Sta II were segregating. On starch gel electrophoresis the Hb Sta II had the same mobility as Hb S and was shown to be an α-chain abnormality but the fingerprints revealed that it differed from Hb G. Three matings in this family were available for study. In one, the husband and wife, both doubly heterozygous for Hb S and Hb Sta II, produced 2 children, one heterozygous for Hb S and one for Hb Sta II. In the second mating, a Hb S trait carrier married a woman doubly heterozygous for Hb S and Hb Sta II. Five children resulted from this marriage: two were heterozygous for Hb S, one was homozygous for Hb S and one doubly heterozygous for Hb S and Hb Sta II, while the fifth was homozygous for Hb S and heterozygous for Hb Sta II. In the third mating, a man doubly heterozygous for Hb S and Hb Sta II who was twice married to normal women, fathered 4 children: two were Hb Sta II trait carriers, one was normal, and one was doubly heterozygous for Hb S and Hb Sta II.

In another family in which abnormal α-chain and abnormal β-chain haemoglobins were found together, Hb S was combined with Hb Hopkins-2 [18], a fast moving haemoglobin that later was shown to be abnormal in the α-chains. In the second generation of this family a critical mating took place between a woman doubly heterozygous for Hb S and Hb Hopkins-2 and a normal man, resulting in 7 children. Of 6 of them studied, one was doubly heterozygous for Hb S and

Hb Hopkins-2, 3 were heterozygous for Hb S and 2 for the fast moving Hb Hopkins-2.

From the findings so far reported and from the additional family described in this paper a close chromosomal linkage between the genes for Hb Gα and Hb Sβ is indeed unlikely

Acknowledgment

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Summary

A second family with Hb G Philadelphia (an α-chain abnormality) and Hb S is described, in which the propositus, a Negro man, was doubly heterozygous for Hb G Philadelphia and Hb S and had 4 major haemoglobins Hb G, Hb S, Hb A, and Hb G/S. His wife was normal. Two of his five children were doubly heterozygous for Hb G and Hb S and had the same haemoglobin pattern as the father; two others were normal, and another child inherited only the gene for Hb G Philadelphia. In this family the interaction between the genes for Hb G Philadelphia and Hb S led to mild anaemia and slight changes in the peripheral blood.

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Effect of Androgen Antagonist on Erythropoietic Action of Testosterone

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It has already been established that the prolonged administration of androgen results in an increase in erythropoiesis either in experimental animals or in patients with various types of aplastic anemia or with breast cancer [1–4] although the exact mechanism remains unknown. Some investigators have recently suggested that androgenic steroid exerts its stimulatory effect on erythropoiesis through increased production of erythropoietin in the kidney [5,6]. One of the authors has pointed out the fact that *this steroid has an ability to enhance a* response of the erythroid tissue to hypoxic stimuli [7]. It is well known that androgen has both anabolic and virilizing activity.

The experiments presented in this paper were carried out in order to clarify the following questions: Which activity is responsible for an increase in erythropoiesis? What relationship exists between erythropoiesis stimulating activity and these two main activities of androgen?

Materials and Methods

Experiment I. Female rats of Wistar strain weighing 120 g were used as experimental animals, and divided into the following 5 groups. Each rat of group 1 received an intramuscular injection of 12.5 mg of testosterone as mixture of enanthate and propionate in 0.1 ml of sesame oil (Enarmon depot®, Teikoku-zoki Co.). Each rat of group 2 was injected with 12.5 mg of methenolone acetate (Primobolan depot®, Schering AG) intramuscularly. Each rat of group 3 or group 4 received an intramuscular injection of 12.5 mg of testosterone or methenolone respectively and thereafter 10.0 mg of antiandrogenic agent, cyproterone acetate* (1,2α-methylene-6-chloro-Δ^{6}-pregnadiene-17α-ol-3,20-dione 17α-acetate) in

* This compound was generously supplied by Dr. F. Neumann, Schering AG, Berlin.

0.2 ml of a benzyl-benzoate-castor oil mixture [1 3(/v)] was injected intramuscularly for week. Group 5 was served as control, which received an intramuscular injection of both 0.1 ml of sesame oil and 0.2 ml of a mixture of benzyl-benzoate-castor oil for week.

Experiment 2. Female mice of ddN-strain weighing 20 to 25 g were made plethoric by two daily intraperitoneal injection with 0.6 ml of an 80 percent suspension of washed homologous erythrocytes. The plethoric mice were divided into 9 groups. Each mouse of group A, B, C, or group D E, F was given the following experimental procedures 6 days after single intramuscular injection with 2.5 mg of testosterone or with both similar dose of testosterone and 10 mg of cyproterone acetate, respectively: intramuscular administration of 0.5 Co-units of purified erythropoietin (Ep) to group A and D exposure to hypoxia of ½ atmosphere for 12 h to group B and E, and intramuscular injections with 0.3 ml of normal saline to group C and F This injection with testosterone or with both testosterone and cyproterone acetate was carried out on the day of the last infusion of erythrocyte suspension. Six days after the last infusion of erythrocyte suspension, each mouse of group G, H or I was given the above described experimental procedures except the injection with testosterone or with both testosterone and cyproterone acetate.

Experiment 3. Seventeen plethoric mice similar to those used in experiment 2 were divided into 4 groups consisting of 4 to 6 mice. Each mouse of group J, K, L or M was injected with 0.6 Co-units of Ep, both 0.6 Co-units of Ep and 10 mg or 5 mg of cyproterone acetate, 0.6 ml of normal saline, respectively

Quantitative determination of erythropoiesis was done as follows rats were injected with 0.5 μc of radioiron ($^{59}FeCl_3$) intravenously 24 h after the last injection of mixture of benzyl-benzoate-castor oil or cyproterone. Mice were similarly injected with 0.5 μc of radioiron intraperitoneally 56 h after the last injection of normal saline, Ep, hypoxic stimulation. The percentage of ^{59}Fe incorporation into red cell mass was determined from the radioactivity of the blood sample taken by cardiac puncture on 24th h in rats and 72nd h in mice after the injection of radioiron, assuming the total blood volume of test animal is 5.0% (in rat) or 7.0% (in plethoric mouse) of the body weight.

Bioassay for erythropoietin was carried out by MIRAND's method [8]. Plasma samples obtained from each animal of the same experimental group were pooled and stored at −20° C until bioassay for erythropoietin activity was done. Activity of the erythropoietin in the pooled plasma sample was expressed as percentage of ^{59}Fe utilized by red cell mass of bioassay mice in 24 h.

Results

1 Effect of androgen antagonist on ^{59}Fe incorporation into red cell in testosterone or anabolic hormone treated rats As shown in table I significant increase in both ^{59}Fe incorporation and reticulocyte counts was noted in rats injected either with testosterone (group 1) or with methenolone (group 2) The activity of erythropoietin in pooled plasma obtained from these animals was evidently elevated, as compared with control rats of group 5. There was, however no significant difference in these values between group 1 and 2. Cyproterone administration did not suppress the enhancing effect of testosterone or methenolone both on ^{59}Fe incorporation into red cell and on erythropoietin activity in

Table I Effect of androgen antagonist on erythropoiesis and plasma erythropoietin level of female rats treated with testosterone or anabolic hormone

Experimental group	No. of animals	% ^{59}Fe incorp. into RBC	Reticulocyte count, %	Plasma erythropoietin level, %
Rats injected with Testosterone (group 1)	5	34.6±5.9	6.26±1.18	4.53±1.38
Methenolone (group 2)	5	31.6±4.5	5.06±0.29	2.21±0.81
Both Testosterone and Cyproterone (group 3)	5	29.6±4.0	5.02±1.44	3.18±1.09
Both Methenolone and Cyproterone (group 4)	5	28.5±5.4	4.30±1.56	2.71±1.02
Normal control (group 5)	8	23.6±4.8	3.5±1.30	0.62±0.21

Mean±S.D. of the mean.

Table II. Effect of androgen antagonist on erythropoietic synergism of testosterone with erythropoietin or hypoxia

Experimental group	No. of animals	% ^{59}Fe incorporation into RBC
Mice pretreated with testosterone		
Erythropoietin (group A)	5	16.20±3.15
Hypoxia (group B)	4	12.38±2.48
Saline (group C)	5	3.65±1.83
Mice pretreated with both testosterone and cyproterone		
Erythropoietin (group D)	5	13.49±2.18
Hypoxia (group E)	4	9.13±2.33
Saline (group F)	5	2.76±1.67
Normal mice		
Erythropoietin (group G)	5	9.50±2.24
Hypoxia (group H)	6	3.44±1.12
Saline (group I)	5	0.42±0.13

Mean±S.D of the mean.

Table III. Effect of androgen antagonist on an enhancing ^{59}Fe incorporation into RBC in plethoric mice induced by exogenous erythropoietin

Experimental group	No. of animals	Hematocrit %	% ^{59}F incorporation into RBC
0.6 Co-U of erythropoietin (group J)	4	63.2±3.7	18.20±2.21
Both 0.6 Co-U of erythropoietin and 10 mg of Cyproterone (group K)	4	59.2±4.4	19.00±2.28
Both 0.6 Co.-U of erythropoietin and 5 mg of Cyproterone (group L)	4	58.5±5.5	16.22±4.00
Saline (group M)	5	62.0±3.0	0.88±0.22

Mean±S.D of the mean.

plasma. Similarly cyproterone exhibited no inhibitory effect on an erythropoietic action of methenolone, as seen in table I

2 *Effect of androgen antagonist on erythropoietic synergism of testosterone with erythropoietin or hypoxia.* The results obtained are shown in table II The combined effect of administration of testosterone with an erythropoietin injection (group A) or exposure to hypoxia (group B) on ^{59}Fe incorporation into red cell exceeded the value calculated from the sum of each stimulus (group C, G and H) From the results obtained in mice of group A and D or group B and E, it was evident that the treatment with cyproterone did not change the synergistic effect of testosterone with erythropoietin or hypoxia on erythropoiesis.

3 *Study of the inhibitory effect of androgen antagonist on erythropoiesis.* The results obtained in experiment 3 are listed in table III Administration of 5 0 or 10 0 mg of cyproterone did not exert influence upon the enhancing ^{59}Fe incorporation into red cell induced by an exogenous erythropoietin given simultaneously

Comment

It is noteworthy that methenolone has the same erythropoietic activity as testosterone at an equal dose level, despite the fact that the virilizing effect was one fourth of that of testosterone. This finding indicates that an erythropoiesis stimulating activity of testosterone may not directly related to its virilizing effect. DUARTE *et al.* have also reported similar

results [9] Cyproterone acetate used in these experiments has no estrogenic effect but blocking activity of androgen receptor in target organ [10, 11]. The results obtained hereabout reveal that cyproterone has no inhibitory effect on erythropoiesis stimulating action of either testosterone or methenolone. An increase in erythropoiesis induced by testosterone should be prevented by this androgen antagonist, if an erythropoietic action of testosterone is related directly to its androgenic effect. There are some evidences indicating that increase in erythropoiesis by testosterone results from an elevation in endogenous erythropoietin activity. Cyproterone did not affect the action of exogenous erythropoietin. Thus, it is indicated from the results presented here that the erythropoietic activity of testosterone is at least independent of its virilizing effect. Administration of androgen antagonist could not inhibit a synergistic effect of testosterone with hypoxia or erythropoietin on erythropoiesis. It is also possible to say that these synergism might not be based upon the virilizing effect of testosterone, although mechanism on these synergism remains to be clarified.

Summary

The mechanism of erythropoietic action of testosterone, especially its relation to virilizing effect was studied, and the following results were obtained. (1) At an equal dose level methenolone has the same erythropoietic activity as that of testosterone. (2) Treatment with sufficient amount of cyproterone acetate failed to suppress an erythropoietic effect of testosterone, methenolone or exogenous erythropoietin. Cyproterone acetate did not prevent significant increase in erythropoietin level in plasma by injecting testosterone or methenolone. (3) Synergistic effect of testosterone with exogenous erythropoietin or *hypoxia on radioiron utilization was not affected by cyproterone acetate. From these results* it is suggested that the erythropoietic action of testosterone is independent of its virilizing effect.

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Maintenance Therapy of Pernicious Anaemia

Comparison of Cyanocobalamin, Hydroxocobalamin and a Cyanocobalamin-Zinc-Tannate Complex

A. Killander and I. Werner

The proper evaluation of different compounds for maintenance therapy in pernicious anaemia has always been difficult. It is well known that following interruption of treatment the time at which a relapse occurs may vary considerably in different patients [19, 15, 9, 4]. In a previous communication we reported the serum B_{12} levels in 45 patients after discontinuation of B_{12} therapy [12]. Twenty patients reached low levels (100 pg/ml and less) within the first year and a further 20 within the second year. One patient was still normal after 26 months whereas the remaining 4 cases did not show low values until 44 months or more after the specific treatment had been discontinued. The reasons for this large individual variation are incompletely known.

The earliest sign of B_{12} deficiency is a subnormal serum level of this vitamin [13, 10]. By taking serial blood samples different B_{12} compounds can be compared, using the patient as the control. The results of such a study are presented in this paper. The substances tested were cyanocobalamin (CN B_{12}), hydroxocobalamin (OH B_{12}) and cyanocobalamin-zinc tannate complex (Zn B_{12}). These abbreviations are used throughout the paper.

Material

The series consisted of 22 patients with untreated pernicious anaemia. In all cases a positive diagnosis was indicated by the presence of the following criteria: macrocytic anaemia, megaloblastic bone marrow, histamine-fast achlorhydria, serum B_{12} concentration of 100 pg/ml and less and a typical response to B_{12} therapy. In 17 cases all 3 B_{12} preparations

were studied consecutively at least once. The remaining 5 patients for different reasons were given only 2 of the compounds. All vitamin B_{12} compounds used contained 1 mg/ml. The following preparations of the above mentioned substances were used: Hepagon forte® (cyanocobalamin) *Redisol®* *Hepagon novum®* (hydroxocobalamin) *Depinar®* (cyanocobalamin-zinc-tannate)[1]

Methods

Routine haematological methods were used. The assay of vitamin B_{12} activity in serum and urine was performed microbiologically using *Euglena gracilis* strain z [8]. In our laboratory values of 100 pg/ml or less are defined as being abnormally low and indicating B_{12} deficiency Values between 100 and 150 pg/ml are regarded as borderline and those between 150 and 900 pg/ml as normal. *Urine was collected for 24 hour periods, kept in refrigerator at +4°C* for not more than 48 h. Frozen aliquots were stored at −20°C until assayed. In some cases ^{57}Co- or ^{58}Co-labelled vitamin were used in the excretion studies. The radioactivity was measured in 1,000 ml volumes in scintillation counter The rate of absorption of the different compounds from the site of injection was studied using radioactive vitamin and surface counting

Each patient received an initial injection of 1 mg of either CN B_{12}, OH B_{12} or Zn B_{12}. Haemoglobin concentration, red cell count, reticulocyte count and serum B_{12} levels were estimated at frequent intervals following the initial injection until normal blood levels were seen. After this period haemoglobin or haematocrit determinations and serum B_{12} levels were performed monthly or in some cases every second month. The interval from the injection to the first low serum B_{12} value was taken as measure of the duration of the therapeutic effect of the substance tested. After the second consecutive low value second injection of another type of B_{12} was given. *The haemoglobin or haematocrit values as well* as the serum B_{12} levels were followed monthly as before. A third injection again of a different substance was given following the finding of two further consecutive subnormal values. In some cases the experiment was continued for up to 3 more injection-relapse cycles.

Results

All 3 preparations were studied in 17 patients. Five of these had CN B_{12}, 5 had OH B_{12} and 7 had Zn B_{12} as their first injection. A full haematological remission was obtained in all patients by this initial injection of 1 mg of vitamin B_{12}. No differences were found between the groups of patients with regard to the time and height of the reticulocyte peak or the rate of increase in the number of red cells. Haematological remission was also maintained during the following experiments and no neurological symptoms or signs developed in any patient. Nor were any other symptoms encountered which could be attributed to B_{12} deficiency

[1] We are greatly indebted to AB Astra, Sweden, Merck, Sharp & Dohme Inc., Rahway N.J., USA and Armour Pharmaceutical Company Kankakee, Ill., USA for the gift of these preparations.

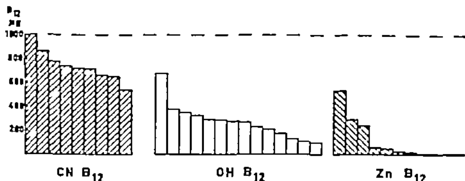


Fig 1 Seventy-two hour urinary excretion of vitamin B_{12} after single injection of 1 mg of CN B_{12}, OH B_{12} and Zn B_{12}. Each block represents 72 hour period in one patient.

Urinary Excretion

The 72 hour urinary excretion of CN B_{12}, OH B_{12} and Zn B_{12} was studied in 9 13 and 11 patients respectively Nine patients were subjected to 2 or 3 excretion studies. The excretion of CN B_{12} and OH B_{12} is negligible after 72 h. In the case of Zn B_{12} the situation is variable. Usually there is a protracted excretion between 72 h and 168 h, in some cases amounting to 5–10%. The results are presented in figure 1 The mean and range of excretion for the 3 substances were CN B_{12} 746 µg (543–1 000) OH B_{12} 284 µg (99–647) and Zn B_{12} 112 µg (3–535) Although there was considerable variation between the different patients it was evident that much more was lost after a single injection of 1 mg of CN B_{12} than of the other 2 compounds tested. About 75% of the CN B_{12}, 30% of the OH-compound and 10% of the Zn B_{12} was lost in the urine during the first 3 days.

In a few cases excretion studies were performed using both an isotope technique and microbiological assay In the case of CN B_{12} alone or in the zinc-tannate complex there was good agreement between the results obtained from the two methods. In some cases that were given OH B_{12} the excretion of radioactivity was considerably higher than the corresponding microbiological activity The reason for this discrepancy was most probably that the urine had been left at room temperature We have found that urine samples left at room temperature will at times cause a fall in B_{12} activity when OH B_{12} is present but not with solutions of CN B_{12}. The figures given for OH B_{12} excretion thus

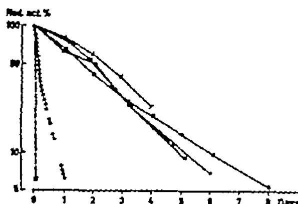


Fig 2. Rate of absorption of 1 mg radioactive CN B_{12} (———) OH B_{12} ( ) and Zn B_{12} (——) from the site of injection.

represent minimum values as it cannot be excluded that some of the urine samples may have been subjected to mishandling as mentioned above.

Rate of Absorption

This was studied in 7 patients. A possibility of a difference between subcutaneous and intramuscular administration was investigated in one patient. One mg of radioactive CN B_{12} was injected intramuscularly in the gluteal region and subcutaneously in the thigh on 2 consecutive days. There was a slight difference in the absorption rate between the 2 routes of administration. Fifty % of the dose had disappeared 42 min after the intramuscular injection and 48 min after the subcutaneous injection. In the other 6 patients the radioactive compounds were only given intramuscularly The absorption rate of the 3 compounds differed considerably as illustrated in figure 2. Thus, 50% of the injected dose of CN B_{12} disappeared 26 and 42 min after the injection (2 cases) The corresponding figures for OH B_{12} was 2.5 h (one case) and for Zn B_{12} 1 6 to 2 4 days (4 cases)

The Serum B_{12} Levels

The time from the injection to the first serum B_{12} level found to be 100 pg/ml and below was considered as the duration of the therapeutic

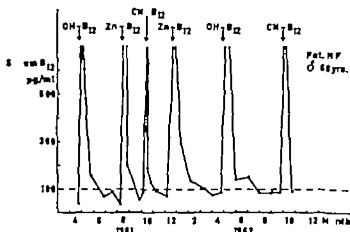


Fig. 3. The serum B_{12} values of case H.F

effect. This level was chosen since all patients, when started on therapy had serum B_{12} values below this level. In order to ascertain that a stable subnormal B_{12} level had been reached the next injection was not given until a second low value had been obtained. In this way the conditions at the time of injection were considered to be as standardized as possible without exposing the patients to the risks of a relapse. It is reasonable to believe that the B_{12} deficiency was most pronounced at the time of the first injection. For this reason the 3 compounds were injected in random order In this way other possible systematic errors were also avoided

The results showed a considerable individual variation, the extremes are represented by patient H. F and K. J Their serum B_{12} levels after the injection of the different compounds are illustrated in figure 3 and 4 In the case of H F the effect of the first CN B_{12} injection did not even last one month. The effect of the long-acting preparations was 2 to 3 months. In the case of K. J the effect of CN B_{12} lasted 2.5 months. After the injection of Zn B_{12} the serum B_{12} level remained above 100 pg/ml for 18 months. After the same amount of OH B_{12} the B_{12} levels were above 100 pg/ml for more than one year when the patient died from a cerebral haemorrhage.

The data from all 22 patients are shown in figure 5 The mean values are given in table I It is evident that the duration of the therapeutic effect of OH B_{12} and Zn B_{12} is considerably longer than

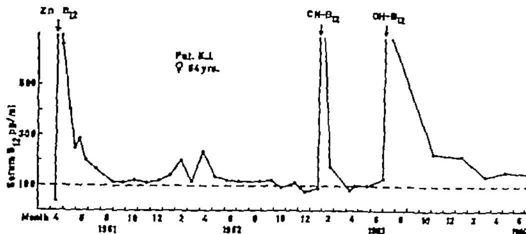


Fig. 4 The serum B_{12} values of case K.J.

Months

S.Ø 1960 · A.P 1962 · S.S. 1963 · K.D 1963 · B.J 1963 · H.P 1963 · L.S 1964 · K.J. 1967

J.K 1967 · S.A. 1966 · L.E 1966 · B.B 1966 · L.K. 1967 · O.C. 1967 · S.B. 1968 · B.K. 1970

CN B_{12} · OH B_{12} · Z B_{12}

Fig. 5. The duration of serum B_{12} levels above 100 pg per ml following injections of 1 mg of CN B_{12}, OH B_{12} and Zn B_{12}. *The preparations are pictured in the order of administration.*

Table I

Substance	No. of injection-relapse-cycles	Duration of cycle in months	
		mean	range
Cyanocobalamin	21	2.1	1–7
Hydroxocobalamin	20	7.1	2–22
Cyanocobalamin-zinc-tannate	22	6.3	2–18

that of CN B_{12}. The results also suggest that the effect of CN B_{12} given as the first injection was much shorter (1 to 2 months) than the effect of the same compound given as second or third injection (1 to 7 months). No significant difference related to the order of injections was found between the other two preparations. The explanation for this prolonged effect of cyanocobalamin was that there probably remained a certain depot effect of the previously given OH B_{12} or Zn B_{12}. A depot effect of this kind might well influence the short period of normal B_{12} level after injections of CN B_{12} but be of no significance in the longer periods following injections of OH B_{12} or Zn B_{12}. However the number of the observations was too small to permit any definite conclusions. No significant differences could be observed between the duration of the effects of OH B_{12} and Zn B_{12}.

Discussion

During the last few years several reports on the therapeutic effect of so-called long acting B_{12}-preparations have been published. The preparation studied are in principle of two types. One is the OH B_{12}, the protracted effect of which is due to its affinity to both specific and unspecific binding proteins in blood and tissues [7]. The other type is represented by preparations in which either zinc-tannate [18] or tannate in a sesame oil/aluminium monostearate gel [16] have been added to CN B_{12} in order to decrease its solubility. In this study only the zinc-tannate-cyanocobalamin complex was used. The cyanocobalamin tannate complex in aluminium monostearate/sesame oil gel (B_{12} TAM®-Dumex) was not available when this investigation was started.

Several reports have been published comparing the effect of OH B_{12} with that of CN B_{12}. It is evident that the urinary excretion of OH B_{12} is much lower than that of CN B_{12} [5, 11 6] The large individual variation found by us has been reported by several authors and there are also considerable differences in the excretion data reported by different laboratories.

The absorption rate from the injection site was studied by GLASS *et al.* [5] and by KILLANDER and SCHILLING [11] *They found a slower* rate of absorption with OH B_{12} compared to CN B_{12}. Our results agree with their findings. In our 4 cases that were given Zn B_{12}, 50% of the radioactivity had disappeared by about 2 days. No other reports on the absorption rate of Zn B_{12} seem to have been published. KRISTENSEN *et al* [14] working with B_{12} TAM found an appreciable activity at the site of injection 2 months after the administration of 1 mg The absorption rate *measured as the disappearance of 50% of the radio*activity was about 20 days. The absorption rate for the TAM preparation thus seems to be extremely slow compared to that of the substances studied in the present investigation.

Serum B_{12} levels after single injections of OH B_{12} have been reported by several authors. They all found much higher levels both initially and after several weeks when compared with CN B_{12} in pernicious *anaemia patients [2 20, 3] After a single injection of 1 mg of* OH B_{12} normal values of serum B_{12} were observed in several patients for periods of 6 to 13 months. The present study clearly demonstrates these differences between the hydroxo- and cyanoforms of the vitamin, although the individual variation is considerable (fig 5) No other reports are available of the effect of Zn B_{12} on the serum B_{12} levels in pernicious anaemia patients. Our results indicate an effect quite *similar to that of OH B_{12}. Studies on the B_{12}* TAM have also shown a protracted effect of the same order of magnitude [1]

It is evident that the urinary excretion of vitamin B_{12} is much less after the injection of OH B_{12} and Zn B_{12} than after CN B_{12}. Both OH B_{12} and Zn B_{12} are able to maintain a normal serum B_{12} level for a much longer time than CN B_{12}. These two points indicate that better results can be obtained with these 2 preparations than with CN B_{12}.

The close similarity of the effects of OH B_{12} and Zn B_{12} is somewhat surprising To judge from the low disappearance rate and the urinary excretion figures a much longer duration of the zinc-tannate compound would have been anticipated. A prolonged urinary excretion of Zn B_{12} observed in a few patients might be one explanation. A defect utiliza

tion of the compound would also explain the above findings. The OH B_{12} represents a more physiological form of the vitamin and it can be assumed that what is retained is also used and metabolized normally in the body Zn B_{12} is an artificial, non-physiological compound of the vitamin and zinc tannate. Without further evidence it cannot be assumed that this highly insoluble material will be completely utilized by the body

Summary

A comparative study was performed on 3 types of vitamin B_{12} cyanocobalamin (CN B_{12}) hydroxocobalamin (OH B_{12}) and cyanocobalamin-zinc-tannate complex (Zn B_{12}) The rate of absorption from the site of injection of CN B_{12}, OH B_{12} and Zn B_{12} measured as the disappearance of 50% of the radioactivity was pproximately 40 min, 2.5 h and 2 days respectively The 72-hour urinary excretion rate after injection of 1 mg of the B_{12} preparation was on the average 75% for CN B_{12}, 30% for OH B_{12} and 10% for Zn B_{12}. A complet haematological remission was obtained in all cases after single injection of 1 mg of all three substances. The serum B_{12} level was maintained bove 100 pg/ml for an average time of 2.1 months with CN B_{12}, 7 1 months with OH B_{12} and 6.3 months with Zn B_{12}. It is concluded that the effect of OH B_{12} and Zn B_{12} is superior to that of CN B_{12}.

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DNA Synthesis by Erythroblasts in Normal Man and in Some Disorders of Erythropoesis

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At present H-thymidine, a specific precursor of DNA, is generally used for the study of the proliferation of the bone marrow cells. Our paper presents investigations on the proliferative activity of the erythroblasts in 13 normal subjects and in 20 patients with marked disorders of erythropoesis.

Materials and Methods

Bone marrow samples were obtained by sternum puncture and were cultivated in liquid medium containing the patient own plasma and Hanck isotonic solution with heparin. ^{3}H-thymidine (specific activity 3.6 c/g) was added to the medium in the end concentration equal to 0.1 μc/ml. The bone marrow suspension in the nutrient medium was carried in 4 tubes in 1 ml and cultivated in shaking incubator at 37° C. I the first tube the incubation was stopped after 15 min, in the other tubes after ½, 1 and 2 h. After the incubation the tubes were centrifugated and smears were prepared from the sediment and fixed with methanol during 10 min. Autoradiography was carried out by the method of Zamorin [21] A liquid nuclear emulsion of the R-type was used

The smears were stained using Boyd's technique [5]. Thirty to 40 young cells (pronormoblasts and basophilic normoblasts) 100 polychromatophilic normoblasts and 50 orthochromic normoblasts were calculated. The percentage of labeled cells was calculated in each group of cells.

The generation time of cells was calculated using the formula of Dombua and Dombua [8] for the exponentially growing population

$$T = \frac{\ln^2}{\ } \quad \text{where} \quad = \frac{\Delta N}{(N \ + N_p) \ \Delta t}$$

T = generation time. ln = 0.693. ΔN = accretion of the number of cells during time t. N = number of cells in period of DNA synthesis. N = proliferative pool, i.e. the ration of cells capable for division and all cells of the given species.

Emulsion R is produced at the Research Chemo-photographic Institute, Moscow (USSR)

Table 1 Clinical and hematological data of 15 patients with hypoplastic and iron deficiency anemia

Pat. No.	Diagnosis	Age years	Sex	Length of disease, years	RBC/mm $\times 10^6$	Hb, g%	FI	WBC/mm $\times 10^3$	Leucocytes % staff cells	segmented neutrophils	lymphocytes	Sedimentation rate mm/h	Reticulocytes, %	Thrombocytes/mm$^3 \times 10^3$	Serum iron, µg%
1	Hypoplastic anemia	48	F	0.3	3.1	11.7	1.1	3.3	2.5	38	59	11	1.8	93	–
2	Hypoplastic anemia	63	M	0.3	3.0	10.6	1.0	4.6	14.5	20	57.5	10	1.2	20	–
3	Hypoplastic anemia	27	F	0.5	1.4	4.35	0.92	2.0	22	17	53	8	–	10	
4	Hypoplastic anemia	19	F	1.2	2.5	8.53	1.0	2.0	10	29	49	20		10	
5	Hypoplastic anemia	14	M	0.2	3.0	11.33	1.1	3.26	1	8	82	20	–	37.5	
6	Chronic posthemorrhagic anemia	29	F	0.75	4.46	9.8	0.67	5.3	0.5	72.5	15	16	2.3	3.79	13
7	Chronic posthemorrhagic anemia	31	M	5	3.9	9.5	0.73	4.7	2	63	25	–	–		
8	Chronic posthemorrhagic anemia	47	F	1	3.5	9.5	0.81	3.5	1.5	51	32	22		318	19
9	Chronic posthemorrhagic anemia	48	F	1	4.8	8.2	0.5	5.5	1	74	11.5	11	0.8	326	
10	Chronic posthemorrhagic anemia	40	F	0.8	4.0	9.2	0.68	6.53	6	63.5	22.5	11	1.2		50
11	Chronic posthemorrhagic anemia	50	F	2	4.4	9.8	0.63	4.6	0.5	69.5	21.5	17			33
12	Chronic posthemorrhagic anemia	34	M	3	5.2	10.5	0.6	5.1	2.5	55.5	32.5	12	1	296	40
13	Essential hypochromic anemia	37	F	1	4.7	10	0.6	5.1	1	59	34	12	0.7	220	70
14	Essential hypochromic anemia	41	F	0.5	4.4	9.4	0.73	4.7	0.5	60.5	30.5	12		240	59
15	Essential hypochromic anemia	46	F	16	3.6	8.5	0.7	4.7	0.5	70	21.5	12	0.2		20

Table II. Clinical and hematological data of patients suffering from polycythaemia vera

at. o.	Age years	Sex	Length of disease years	RBC/ mm³ ×10⁶	Hb, g%	WBC/ mm³ ×10³	Thrombocytes/mm³ ×10³	Sedim. rate mm/h	Viscosity of blood	Hematocrit %	Increase of liver	Increase of spleen
	39	M	1	6.44	21.0	13.0	320	2	16.6	83/17	+	+
	48	M	14	8.1	18.3	7.2	130	1.5	8.5	70/30	+	+
	42	F	2	6.08	17.6	14.2	600	2	10	80/20	+	+
	58	M	3	6.95	21.0	29.2	400	2	12.5	79/21	+	+
	34	F	5	7.25	23.6	4 4	170	1	16.6	88/12	+	+

It was assumed in the calculations that N t constant concentration of ³H-thymidine is equal to 1 i.e. all cells after period of DNA synthesis are divided and their destruction is minimal.

Control subjects were donors selected for bone marrow transplantations. Among the patients 3 had hypoplastic anaemia 5 polycythaemia vera and 10 iron-deficiency anaemia (7 chronic posthemorrhagic and 3 essential hypochromic anaemias). The clinical and hematological data of the patients are shown in tables I and II.

Results

1 Normal subjects After 15 min incubation of bone marrow in the presence of ³H-thymidine the percentage of the label in pronormoblasts and basophilic normoblasts was 76.6 ± 0 73 and in polychromatophylic normoblasts 23 0 ± 0 86. After 2 h incubation there was a significant increase of the label ($p < 0.01$) up to 88.8 ± 1 1 and 37.6 ± 2.4 correspondingly (orthochromic normoblasts did not incorporate ³H thymidine)

The generation time of pronormoblasts and basophilic normoblasts was 17.8 ± 0.94 h, and that of polychromatophilic normoblasts 11.2 ± 1 1 h.

2. *Hypoplastic anemia.* After 15 min of cultivation the percentage of the label was 31.5 ± 6.06 in pronormoblasts and basophilic normoblasts and 8.2 ± 2.04 in polychromatophilic normoblasts. After 2 h the percentage increased up to 66.5 ± 5 03 and 14 6 ± 1.34 correspondingly

The generation time of pronormoblasts and basophilic normoblasts was 4.9 ± 0 6 and that of polychromatophilic normoblasts 17 6 ± 3.72 h.

3 *Polycythaemia vera.* After 15 min cultivation the percentage of the label was 68.2 ± 1 87 in pronormoblasts and basophilic normoblasts and 19 7 ± 2.45 in polychromatophilic normoblasts. After 2 h it was 85.5 ± 2.93 and 33 3 ± 4 1 correspondingly

The generation time of pronormoblasts and basophilic normoblasts was 11 7 ± 1 2 h, and in polychromatophilic normoblasts it was 11 6 ± 0 9 h.

4 *Chronic posthemorrhagic anemia.* After 15 min cultivation the per centage of the label was 69 8 ± 3 66 in pronormoblasts and basophilic normoblasts and 24.3 ± 3 65 in polychromatophilic normoblasts. After 2 h incubation the label was 86.9 ± 1 9 and 32.5 ± 2.6 correspondingly

The generation time of pronormoblasts and basophilic normoblasts was 12.7 ± 1.5 h and that of polychromatophilic normoblasts 17.5 ± 3.39

5. *Essential hypochromic anemia.* After 15 min incubation the per centage of the label was 76.3 ± 2 0 in pronormoblasts and basophilic normoblasts and 22.0 ± 0.26 in polychromatophilic normoblasts. After 2 h it was 87 6 ± 3 9 and 31 6 ± 1.2 correspondingly

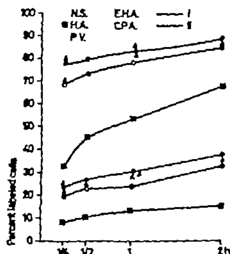


Fig 1 Uptake of ³H-thymidine in erythrocytic precursors of healthy subjects and patients with some disorders of erythropoesis. ● normal subjects, ■ hypoplastic anemia, ○ polycythaemia vera, △ essential hypochromic anemia, ▲ chronic posthemorrhagic anemia. —— pronormoblasts and basophilic normoblasts. polychromatophilic normoblasts.

The generation time of pronormoblasts and basophilic normoblasts was 22.7 ± 5.83 h and in polychromatophilic normoblasts 16.0 ± 4.16 h.

All results are demonstrated graphically in figure 1

Discussion

The results of the present study in normal subjects are in agreement with those of other authors [1–3 6 7 11 13 16 19 20]

There are only few publications on the DNA synthesis of bone marrow cells in hypoplastic anemia [1 9 10 12, 14 15 17] According to these results our studies indicate a decrease of the cells synthesizing DNA and of the intensity of DNA synthesis. The marked fall of labeled cells after 15 min incubation also illustrates the decrease of DNA producing cells in the bone marrow of patients with hypoplastic anemia. The observation during 2 h shows that in these patients the increase of the label of pronormoblasts and basophilic normoblasts is 3 times greater than in normal subjects. However the label does not reach the normal values by the end of incubation. The high increase of the labeled pronormoblasts and basophilic normoblasts can be explained by a shortened presynthetic period G in the mitotic cycle of these cells, that is followed by an increased proliferative possibility of a small part of pronormoblasts preserving their ability for DNA synthesis. A marked shortening of the generation time confirms this assumption.

In hypoplastic anemia the slight increase of labeled polychromatophilic normoblasts and the more prolonged generation time may signify a prolongation of the presynthetic G period and, possibly of DNA synthesis.

The significant decrease of the label ($p < 0.01$) after 15 and 30 min incubation of the bone marrow of patients with polycythaemia vera confirms a diminution of the cells taking part in proliferation. It is evident that a considerable part of cells is in the 'rest period (G_0-period) The appearance of substances like inhibitors [8] or chalones [4] in the bone marrow of these patients cannot be excluded. These substances are contained in mature erythrocytes and prevent an entry of young cells into the mitotic cycle. By the end of the incubation the percentage of the label is almost normalized, that indicates the accelerated passing of cells through all periods of the mitotic cycle. This is

confirmed by a shortening of the generation time of pronormoblasts and basophilic normoblasts. The shortening of the mitotic cycle of pronormoblasts and basophilic normoblasts may be one of the factors that lead to the pathological increase of erythrocytes in polycythaemia vera.

There is no significant difference in the percentage of labeled cells between normal subjects and patients with iron deficiency. Thus there is no disturbance of DNA synthesis of the erythroid cells of patients with iron deficiency anemias. At the same time the generation time of polychromatophilic normoblasts is increased.

It is evident that the latter depends on the delay of polychromatophilic normoblasts in the presynthetic period (G_1) that may be stipulated by the disturbance in hemoglobinisations. It is of interest, that the generation time of pronormoblasts and basophilic normoblasts in essential hypochromic anemia was 10 h longer than in chronic posthemorrhagic anemia.

Summary

The DNA synthesis of the erythroid cells was investigated in bone marrow cultures by an autoradiographic method using ^{3}H-thymidine. A decrease of the label was found in hypoplastic anemia indicating a considerable diminution of the proliferative pool of erythroid cells. There was a slight decrease of labeled cells in polycythaemia vera at the beginning of incubation indicating some diminution of proliferation. However by the end of incubation period the percentage of the labeled cells was normalized. In iron deficiency anemias the ^{3}H-thymidine incorporation did not differ from that in normal subjects.

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Change from Fetal to Adult Hemoglobin in Relation to Changes in the A Antigen of Human Erythrocytes

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Fetal erythrocytes differ from those produced in adult individuals not only in size and in the type of hemoglobin, but also in antigenic properties of the cell membrane. Fetal erythrocytes possess an antigen that is no longer present in red cells of adults [6] and the ABH antigens are weaker in fetal red cells than in those of adults. Strength or reactivity of the A antigen increases during fetal life and infancy until adult values are reached at 2 to 3 years of age, thereafter remaining relatively unchanged for the rest of the individual's life [2]. Change of the A antigen therefore overlaps the change from fetal to adult hemoglobin that takes place during the last trimester of gestation and early infancy. At birth, both the A antigen and hemoglobin are in process of undergoing change.

The question arose as to whether change in the A antigen and change in hemoglobin are closely interrelated. This was determined by utilizing blood samples of newborn infants and multivariate analyses. Results on the interrelation between strength of the A antigen and percentage of adult hemoglobin, as well as findings on effects of the infant's sex and birth weight are presented in this report.

Materials and Methods

Freshly collected samples of blood from the umbilical cord were used. Strength of the A antigen and blood group subtypes of A were determined as previously described [3, 4].

A modification of the technique of SINGER *et al.* [8] was used for determining fetal hemoglobin. *KOH solution was used at 1/10 N*, and the 1 N solution was stored at 4°C. The $(NH_4)_2SO_4$ solution was saturated at 25°C, diluted to 75% saturation, and also stored at

4 C. Washed and packed red cells were hemolyzed with distilled water and toluene. After centrifugation, the 2 upper layers were discarded and the red solution filtered through double Whatman No. 2 paper filter With the filtrat and distilled water hemoglobin solutions were prepared that gave reading of exactly 500 units in Klett-Summerson colorimeter with green filter (corresponding to O.D. = 1.00) Three test tubes (size 13×100 mm) each with 1.5 ml of the standardized hemoglobin solution, were prepared from each sample of blood. Temperature was standardized to 25°C in constant temperature water bath. T each tube 1.0 ml of 1/10 N KOH, which had been prewarmed to 25 C, was added at determined intervals. After exactly 5 min of incubation, 2.0 ml of cold $(NH_4)_2SO$ solution was added to each tube. The tubes were inverted twice and the content filtered through dry No. 2 Whatman paper filter Filtration was repeated through the same filter at 15 min after the addition of $(NH_4)_2SO_4$. Optical density was determined twice first, after the second filtration t 30 min after the addition of $(NH_4)_2SO$ and again, after the third filtration at 45 min after the addition of $(NH_4)_2SO_4$. Percentage of fetal hemoglobin of sample was computed from the mean of the 6 readings (3 tubes measured twice) and the values converted to adult hemoglobin (100 minus percentage of fetal hemoglobin = adult hemoglobin).

Correlation coefficients were used, as more meaningful presentation of the relative magnitude of association between variables is possible than with regression coefficients. Partial correlation coefficients, form of multivariate analyses, have been described, for example, by Kendall and Stuart [7].

Percentage of fetal hemoglobin was determined in cord blood samples of 97 infants. Only 86 infants, however could be included in the analysis as 6 mothers were uncertain about their estimated date of conception; 5 non-Caucasians were not included because of known racial differences in birth weight and development [5]. The strength of the A antigen was determined in 54 A_1 Caucasian infants born to mothers of blood type A. Two girls, one resulting from 250 and an other from 297 day pregnancy represented the extremes in duration of pregnancy among the infants in whom strength of the A antigen had been determined. Duration of gestation ranged from 210 days (boy) to 305 (girl) among the infants in whom fetal hemoglobin had been determined.

Results

Figure 1 shows the frequency distribution for percentage of adult hemoglobin in the 86 infants and strength of the A antigen in the 54 A_1 infants. The figure reveals wide variation between infants. By coincidence the values for percentage of adult hemoglobin and strength of the A antigen are of similar magnitude.

In 24 infants, both strength of the A antigen and percentage of adult hemoglobin were determined. The results, illustrated in figure 2 reveal a positive association between the two variables. The simple correlation coefficient between strength of the A antigen and percentage of adult hemoglobin was $r = 0.52 \pm 0.12$ which, for 22 DF is highly significant ($P < 0.01$) Part of this association might result from a common effect of duration of gestation or a common effect of weight. These effects were corrected with partial correlation analyses.

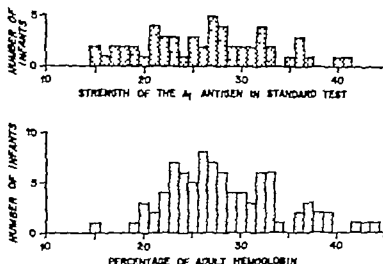


Fig. 1 Frequency distribution of strength of the A antigen and percentage of adult hemoglobin in newborn infants.

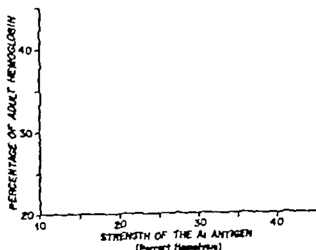


Fig. 2 Association in infants in whom both strength of the A antigen and percentage of adult hemoglobin were determined. The dots represent individual values.

The partial correlation coefficient (holding both birth weight and duration of gestation constant) was $r_{AH \cdot WD} = 0.44 \pm 0.18$ for 20 DF this is significant ($P < 0.05$). Thus, there remains a significant association between *strength* of the A antigen and percentage of adult

Table I Mean percentage of adult hemoglobin and mean strength of the A antigen in newborn infants

Sex	Number of infants	Mean duration of gestation days	Mean birth weight kg	Mean percentage of adult hemoglobin
Boys	50	278.4 ± 1.9	3.36 ± 0.55	27.49 ± 0.80
Girls	56	283.8 ± 1.7	3.34 ± 0.43	29.54 ± 1.00
				Mean strength of the A antigen
Boys	31	280.9 ± 1.3	3.[illegible]0 ± 0.48	24.78 ± 1.11
Girls	23	278.3 ± 2.4	3.44 ± 0.44	28.62 ± 1.52

Strength determined in immunohemolytic test

hemoglobin that is independent of birth weight and of duration of gestation.

Means and standard errors for percentage of adult hemoglobin and strength of the A antigen are presented in table I for boys and girls separately. Percentage of adult hemoglobin was slightly higher in girls than in boys but the difference was not statistically significant ($P > 0.05$). Duration of gestation was significantly lower in boys than in girls and this accounts in part for the observed higher mean percentage of adult hemoglobin in girls than in boys. A slight difference, however was still discernible among the infants resulting only from pregnancies of 270 to 290 days duration the mean percentage of adult hemoglobin for girls was 29.7 ± 1.23 and that for boys was 28.0 ± 0.96

Strength of the A antigen, on the other hand was significantly higher in girls than in boys ($P < 0.05$). As table I shows, the average duration of gestation was slightly higher for the 31 boys than for the 23 girls, i.e., an even larger difference in antigen strength may be expected for equal duration of gestation.

Simple and partial correlation coefficients are presented in table II. The table shows that the simple correlation coefficient between percentage of adult hemoglobin and duration of gestation was slightly higher than that between percentage of adult hemoglobin and birth weight. Both were highly significant ($P < 0.01$). The partial correlation coefficients, holding either duration of gestation or birth weight constant, were markedly lower than the corresponding simple correlation coefficients.

Table II. Simple and partial correlation coefficients of percentage of adult hemoglobin and strength of the A antigen with birth weight and duration of gestation

		Birth weight (W)	Duration of gestation (G)
Percentage of adult hemoglobin (H)	Simple corr coeff.	$= 0.34^{1} \pm 0.10$	$r = 0.38^{2} \pm 0.09$
	Partial corr coeff.	$r_{HW.G} = 0.18 \pm 0.11$ (holding duration of gestation constant)	$r_{HG.W} = 0.24^{1} \pm 0.10$ (holding birth weight constant)
Strength of the A antigen (A)	Simple corr coeff.	$r = 0.42^{2} \pm 0.11$	$= 0.21 \pm 0.13$
	Partial corr coeff.	$_{AW.G} = 0.31 \pm 0.13$ (holding duration of gestation constant)	$_{AG.W} = 0.05 \pm 0.14$ (holding birth weight constant)

[1] $P < 0.05$ [2] $P < 0.01$

Table II further reveals that strength of the A antigen is more closely correlated with birth weight than with duration of gestation, the partial correlation coefficient between strength of the A antigen and duration of gestation being close to zero. The partial correlation coefficients thus show a trend in the opposite direction: strength of the A antigen was closer correlated with birth weight than with duration of gestation, while percentage of adult hemoglobin showed a slightly closer association with duration of gestation than with birth weight.

Discussion

Newborn infants revealed wide variation in strength of the A antigen of red cells and in percentage of adult hemoglobin. The analyses disclosed that part of this variation resulted from variation in duration of gestation and variation in birth weight. However, holding duration of gestation and birth weight constant in partial correlation analysis, still revealed a significant association between strength of the A antigen and percentage of adult hemoglobin. An association between strength

of the A antigen and percentage of adult hemoglobin was thus demonstrated that is independent of variation in duration of gestation and variation in birth weight. Despite this association, the results have also provided evidence that change in the A antigen and change in hemoglobin are not fully interdependent. This was demonstrated by the following findings (a) the correlation between strength of the A antigen and percentage of adult hemoglobin was less close than could be expected if the change were fully synchronized (fig 2) (b) strength of the A antigen was much closer correlated with birth weight than with duration of gestation, while percentage of adult hemoglobin was slightly closer correlated with duration of gestation than with birth weight (c) an apparent effect of sex was more marked for strength of the A antigen than for percentage of adult hemoglobin (d) change in hemoglobin is completed at an age before strength of the A antigen has reached adult values [2] These findings suggest that change in the A antigen and change in hemoglobin may be controlled by different factors.

The partial correlation coefficients determined in the present study may be used to provide a measure of the causes that affect the change in hemoglobin and the change in the A antigen, in particular to determine whether change is predominantly a function of chronological age or of the infant's development (expressed in birth weight) Either of the two might be feasible. Duration of gestation, however was found to be an almost negligible determinant for the A antigen when compared with the effects associated with birth weight. The significant partial correlation coefficient between strength of the A antigen and birth weight (holding duration of gestation constant) suggests that relative development of the infant (expressed in weight) has a marked effect on the change in the A antigen. In general, rapidly developing infants (expressed in birth weight) are expected to show a relatively faster increase in strength of the A antigen than slower developing infants. This conclusion is further supported by the finding of a higher strength of the A antigen in girls than in boys. Girls are known to develop faster than boys (physiologic, skeletal) despite the fact that boys exceed girls in size and weight [1 9] In connection with properties of the erythropoietic system, the faster skeletal development in girls than in boys, as reported by CHARTTE [1] is of particular interest.

In contrast to the A antigen, percentage of adult hemoglobin was found to occupy more an intermediate position in its association with *duration of gestation and birth weight. The significant partial corre*lation coefficient between duration of gestation and percentage of adtul

hemoglobin indicates that the change from fetal to adult hemoglobin is markedly influenced by chronological age of the infant. Thus, change in hemoglobin seems to be more dependent on time than on the infant's weight, while for the A antigen the reverse is the case.

The significant partial correlation coefficient between strength of the A antigen and birth weight indicates also that weight is not merely an expression of size, but may provide information on the relative development of the infant. This has long been known to be valid in cases of prematurity. The present results reveal, however, that this is applicable even for infants resulting from pregnancies of the same duration.

Summary

Strength of the red cell A antigen and level of adult hemoglobin were found to be significantly correlated in newborn infants, even after correction for variation in birth weight and duration of gestation. Furthermore, strength of the A antigen was significantly higher in girls than in boys and much closer associated with birth weight than with duration of gestation. On the other hand, percentage of adult hemoglobin was somewhat closer associated with duration of gestation than with birth weight. The results indicated that relative development of the infant (expressed in birth weight) has marked effect on the change in the A antigen, while the change in hemoglobin is more dependent on chronological age of the infant.

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Congenital Deficiency of Fibrin Stabilizing Factor (Factor XIII)

A Report of the First Case from Latin America

T. Arends, J. M. Guevara and J. R. Rangel

The cases of congenital deficiency of the fibrin stabilizing factor (FSF) (synonyms: factor XIII, Laki and Lorand factor, fibrinase, Atoka diathesis) described to date have been found in Europe, Switzerland [1], Finland [2, 3], France [4, 5], Italy [6, 7], England [8], Greece [9] and Denmark [10]. It has also been reported in Israel [11], in the United States [12–13] and Canada [14]. No cases have been previously reported in Latin America.

The case reported here concerns a FSF deficiency studied in a Venezuelan native which was classified as congenital by the results obtained from clinical and laboratory tests.

Methods

The coagulation tests were performed by the standard methods [15], and the plasma clot solubility in 5 M urea was done according to Losowsky *et al.* [8]. The defect of these patients being in the clot, it was thought that re-dissolving it with trypsin and studying it electrophoretically a difference might be observed when compared with the normal subjects. The following method was used: 0.5 ml of oxalated plasma mixed with 0.5 ml of 0.025 M of $CaCl_2$, incubated 30 min at 37 °C; the clot was tapped loose and washed 3 times with NaCl 0.85%, and then trypsin digested with a drop of commercial trypsin (NBCo) 4 mg/ml at 37 °C for 2 h, shaking gently at intervals 2 or 3 times; trypsin digestion was stopped adjusting to pH 6.4 with 0.5 N acetic acid, and centrifugated at 2000 rpm. Acetate cellulose electrophoresis of the supernatant liquid was made applying the sample 6 times.

hemoglobin indicates that the change from fetal to adult hemoglobin is markedly influenced by chronological age of the infant. Thus, change in hemoglobin seems to be more dependent on time than on the infant's weight, while for the A antigen the reverse is the case.

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Table I. Effect of the mother's plasma on the patient's clots. Incubation of the clots in 5M urea, expressed after B... and DELAGE [14]

Subject studied	Correction power of plasma added *in vitro*							
	Pure	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Control	++	+–	– –	–	– –	–	=	0
Mother	++	+–	– –		–	0	0	0

and indirect platelet count were all normal. The solubility of the recalcified plasma clot was demonstrated in 5M urea solution. Other hematologic and biochemical tests were normal, except the hemoglobin which was somewhat low: 10.1 g/100 ml. Peripheral blood smear showed mild hypochromia of the red cells.

Physical therapy was instituted, consisting of moderate exercises and electrostimulation of the right anterior tibial muscles.

A year after she was discharged, the patient was hospitalized in coma in her home town and died within 24 h. Post mortem examination revealed intraparenchymatous cerebellar hemorrhage with rupture of the upper side of the right cerebellar lobe.

Electrophoresis of the recalcified plasma clot. The electrophoresis of the trypsin digested clot revealed, in addition to the albumin and fibrinogen, 2 other fractions migrating ahead of the albumin. Other minor fractions, slower than albumin, were not identified (fig. 1). The patient's trypsin digested clot and that of her mother's, did not show difference when compared with the results obtained in normal subjects.

Family studies. The solubility of the plasma clot in 5M urea was done to the mother and maternal uncle, not finding abnormalities in either one. The capacity of correction of the mother's diluted plasma, done according to JOSSO *et al.* [3] was slower than the normal control (table I). This method has been used to determine heterozygous subjects.

Discussion

MACFARLANE [16] estimated the probable incidence of FSF deficiency to be 0.1 × 10 inhabitants, which makes it one of the coagulation inborn errors of lowest frequency. Nevertheless, the possibility of mistaking it with the hemophilias should be kept in mind when dealing with hemophiloid states. The fact that to this writing only one case has been found in Venezuela – with a population of more than 9,500 000 – apparently confirms the reported low incidence of this disorder.

The genetic mechanisms of this condition has been considered as determined by a recessive autosomic gene although abnormal results found in the plasma of parents of some patients with congenital deficiency of FSF [1, 5, 13, 14, 17] allows the postulation of a possible

Table II Clinical characteristics of the published

Case	Sex	Age years	Country	Parents consang.	Other family memb. studied deficients	non-deficients
1	M	7	Switzerland	yes	1 brother (case 2) 1 cousin	2 great-uncles 2 cousins
2	M	18m	Switzerland	yes	case 1	–
3	M	2	Finland	no	?	?
4	M	6	France	no		1 brother
5	F	2	Italy	yes	case 18	–
6	M	14m	France	no	mother	–
7	M	4	France	yes	parents[3]	
8	F	32	Finland	yes	–	
9	M	26	USA	no	1 brother (case 10)	
10	M	23	USA	no	case 9	
11	M	12	Canada	no	parents	
12	M	28	England	no	–	
13	M	4	England	no	–	
14	M	3	England	no		
15	M	24	Greece	?		
16	M	12	Denmark	no		parents
17	F	29	Israel	yes	–	
18	F	1	Italy	yes	1 sister (case 5)	parents
19	M	3	USA	no	2 siblings parents grand-mother[3]	maternal grand-fathers[3]
20	F	5	USA	no	parents 2 sisters 1 brother[4]	
21	F	20	Venezuela	no	mother[3]	

[illegible] Age at the time of diagnosis
[illegible] Plasma clot was soluble in 5M urea
[illegible]

cases of congenital deficiency of FSF (Factor XIII)

Bleeding manifestations					Hemostat.		Authors
umbilical	ecchymoses	hematomas	dental	other	Cicatrisat.	eff. of transf.	
+	+	+	—	Post wounds, subdural hematoma	slow	yes	Duckert *et al.*, 1961 [1]
+	+	+	—			?	
—	+	—	—	post wounds			Ikkala and Nevanlinna, 196. [2]
+	+	+	—	post wounds		yes	Marche, 1963 [4]
+	+	+	+	post wounds		yes	M ndelli 1963 [6]
+	+	+	—	otorrhagia, cerebro-meningeal		yes	Josso *et al.*, 1964 [5]
—	+	+	—	post wounds		yes	
+	—	—	—	hemoperitoneum, hemarthrosis		yes	Ikkala *et al.*, 1964 [3]
	+	+	+	epistaxis	slow	yes	Hampton *et al.*, 1965 [12]
?	+	+	+	epistaxis		yes	
—	+	+	—	hemarthrosis, subdural hematoma		yes	Barr and Delage, 1965 [14]
—	+	+	—	hemoperitoneum		yes	
+	+	+	—			yes	Losowsky *et al.*, 1965 [8]
+	+	+	—			yes	
—	—	—	—	post peri-dectomy post wounds	slow		Trevisani *et al.*, 1965 [9]
—	+	+	—	post wounds, hemarthrosis, subdural hematoma	slow	yes	Amris and Ranek, 1965 [10]
+	+	+	+	abortions, hematuria	slow	yes	Fisher *et al.*, 1966 [11]
—	+	+	+	post wounds		yes	Ott zani *et al.*, 1966 [7]
—	+	+	—	post wounds, hemarthrosis		yes	Britten, 1967 [15]
+	+	+	—	post wounds		yes	Bouhasin and Alta 1968 [17]
+	+	+	—	post wounds, cerebellar hemorrhage		yes	this report

Partial deficiency of FSF demonstrated by thromboelastography
Platelets transamidase test
FSF levels

J. H. P. Jonxis und P. H. J. Huisman: **A Laboratory Manual on Abnormal Haemoglobins.** 2nd ed. Blackwell, Oxford/Edinbourgh 1968. 126 p. Price 30 s.

This manual was, as stated by the authors, 'primarily written for the medical pathologists and the laboratory workers receiving samples of blood for investigation, but who have not made special study i this field' Indeed the book is clearly and concisely written, both in its initial part on clinical data and in the subsequent chapters on biochemical evaluation of bnormal hemoglobins. Much practical information effects the ast personal experience of the authors, and many useful details are given which cannot b easily found in the original literature. The procedures include only few and important references to original publications which facilitates reading. I the reviewer opinion, there is too much referring from one chapter to another. On page 48 the authors state that 'the elution rates (on DEAE-Sephadex) are similar to those seen in DEAE cellulose chromatography but in the corresponding section the reader again is referred to CM-chromatography concerning the same question. The reader is also supposed to be familiar with migration patterns of abnormal haemoglobins on electrophoresis (g. plates 3 and 4). The later chapters cannot be well understood without the complet reading of the earlier parts of the book. In general, the second edition of the Laboratory Manual on Abnormal Hemoglobins' is most welcome gift t every investigator interested in haemoglobinopathies. *E. Beck, Basel*

H. S. Cox: **Medical Cyto-Technology.** Butterworth, London 1968. 63 p. Price 10 s.

The small monograph is concerned exclusively with exfoliative cytology and describes the current diagnostical methods including the technique for obtaining and preparing specimens of vaginal, cervical and endometrial smears, bronchial aspirates, gastric washings and ascites fluids. The staining techniques are reported and the characteristics of normal and malignant cells as well as the problems of cyto-screening are briefly discussed. Some recommendations for laboratory training are given t the end of the booklet, which offers concise introduction in cytology for students, general pathologists and technicians and also stimulates more detailed studies necessary for the specialized cytologist.

H.R. Marti, *Aarau*

L. H. B. Walley and J. R. Robinson: *Human Haemoglobin Variants and their Identification.* Butterworth, London 1968. 56 p. Price 10 s.

The booklet contains in its first 15 pages brief introduction in the field of haemoglobinopathies with summaries on the haemoglobin molecule, the genetic code, the abnormal haemoglobins, the thalassaemias, the Hb M variants and the inheritance and geographical distribution of the most frequent abnormalities. In these short chapters many details had to be omitted, only small selection of abnormal haemoglobins are mentioned and the heterogeneity of thalassemia is incompletely reported. In the main part of the booklet the current laboratory techniques are described ith details necessary to perform the methods. Different techniques for detection of inclusion bodies, the sickle cell test, several methods of lkali denaturation, the technique of electrophoresis on cellulose acetate starch gel and block, agar gel, polyacrylamide gel and paper the ion exchange chromatography the heat denaturation, Hb S solubility test and the stain for Hb F in red cells are given. This small olume is certainly valuable help for pathologists and technicians.

H. R. Marti, *Aarau*

S. [illegible]. **Die Thrombozytentransfusion.** Untersuchungen mit radioaktiv markierten Thrombozyten. Fischer, Stuttgart 1968. 119 S., 32 Abb. Preis DM 29.50.

Plättchentransfusionen haben im Laufe der letzten Jahre an Bedeutung gewonnen, da von klinischer Seite her, besonders im Zusammenhang mit der intensiveren zytostatischen Behandlung der Neoplasien, die Nachfrage stark gestiegen ist. Das vermehrte Interesse spiegelt sich auch in der rasch zunehmenden Flut der diesem Thema gewidmeten Literatur. Ein Überblick in der Art der vorliegenden Monographie kommt deshalb sicher einem allgemeinen Bedürfnis entgegen. Dabei hat der Autor sich die Sache nicht leicht gemacht: er begnügt sich nicht mit der Zusammenstellung der Literatur, sondern er hat versucht, die verschiedenen Ansichten und die noch offenen Fragen – und es sind deren viele – selbst zu überprüfen und mit besonderen Versuchsanordnungen einer Lösung näher zu bringen. Mit Recht legt der Autor für die Beurteilung der Funktionstüchtigkeit der Plättchen das Hauptgewicht auf die Überlebenszeit radioaktiv markierter Thrombozyten. Ein ausführlicher erster Teil ist der Methodik der Thrombozytenmarkierung (^{51}Cr und $DF^{32}P$) und allen damit zusammenhängenden Problemen gewidmet. Unter den für die Beurteilung der Thrombozytenfunktion herangezogenen in-vitro-Methoden verdient vor allem die vom Autor erstmals auf Thrombozyten angewandte Zellelektrophorese Beachtung. Im Zusammenhang mit der Herstellung von Thrombozytenkonserven hat der Autor die wichtige Frage des geeignetsten Antikoagulans noch einmal aufgegriffen und mit Hilfe der in-vivo-Methoden überprüft. Dabei konnte er die Überlegenheit der ACD-Lösungen mit niedrigem pH bestätigen. Zur Konservierung der Thrombozyten wird anhand eigener Versuche Stellung bezogen. Die Besprechung der klinischen Indikationen der Plättchentransfusion wird zum Anlass genommen, um Fragen des Thrombozytenumsatzes bei den verschiedenen Thrombopenieformen eingehend zu diskutieren. Im Abschnitt über die Thrombopenien als Folge eines vermehrten Verbrauchs finden sich interessante Beiträge zu den postoperativen Thrombopenien; die eigentlichen Verbrauchskoagulopathien, die regelmässig mit einer Thrombopenie einhergehen, finden jedoch keine Erwähnung. Der letzte Abschnitt ist immunologischen Aspekten der Thrombozytentransfusion gewidmet, wobei der Kreis so weit gezogen ist, dass fast das ganze Gebiet der Thrombozyten-Immunologie Berücksichtigung findet. Neben der Gegenüberstellung (und Wertung) der verschiedenen Methoden zum Antikörpernachweis scheinen uns die Untersuchungen zur Frage der Spezifität der Isoantikörper besonders wertvoll. Beachtung (und Nachprüfung) verdient sodann die Feststellung, dass das Serum von ITP-Patienten die elektrophoretische Beweglichkeit normaler Thrombozyten regelmässig zu vermindern vermochte. Der (hypothetische) «Autoantikörper» verhält sich offenbar in dieser Hinsicht anders als die Isoantikörper, welche in der Regel nicht zu einer Beeinflussung der elektrophoretischen Beweglichkeit führten. Im übrigen hat der Autor die Erfahrung anderer bestätigt, dass die Überlebenszeit der Plättchen den zur Zeit feinsten Hinweis auf das Vorliegen von Antikörpern zu geben vermag. Von praktischer Bedeutung ist schliesslich der Nachweis, dass Hämolysine oder Agglutinine des ABO-Systems mit erhöhtem Titer zum beschleunigten Abbau ABO-inkompatibler Plättchen führen.

Die Monographie vermittelt eine Fülle von höchst interessanten und wichtigen Daten aus dem Kapitel der Thrombozytenphysiologie und -pathologie. Bei der Vielschichtigkeit des Gebotenen mag der Titel des Buches sogar etwas zu bescheiden ausgefallen sein. Andererseits vermisst der praktisch orientierte Leser vielleicht eine verbindliche Empfehlung, wie im Einzelfall für die Thrombozytentransfusion vorzugehen sei, z.B. in Bezug auf Wahl des Präparates (PRP oder Konzentrat), technische Durchführung der Konzentration unter Berücksichtigung aller diskutierten Erkenntnisse, Anzahl der benötigten Spender, Möglichkeiten der Thrombopherese, usw. Das Buch wird aber von jedem, der sich mit den Thrombozyten beschäftigt und der sich über die Möglichkeiten der Plättchentransfusion orientieren möchte, mit Gewinn gelesen werden. Die klare Gliederung erleichtert die Lektüre, wobei wir die am Ende jedes Abschnittes gegebene Zusammenfassung als besonders angenehm hervorheben möchten.

U. Bucher, *Bern*

sonian pernicious anaemia also showed a normal TKA of the red cells. It has been found earlier that carriers of broad fish tapeworm may exhibit disorders of folic acid metabolism [8, 12, 14 22] This might be the reason why the enzyme metabolism of the red cells, in at least some anaemic tapeworm carriers, does not follow the same course as in Addisonian pernicious anaemia. The same might also be suggested by the results obtained from the folic acid deficiency patients of the present series (table IV No. 1–3) Levitan et al. [10] found on serum lactate dehydrogenase, that its activity is increased in Addisonian pernicious anaemia but in no other loblastic anaemias. It is true that later observations have shown increased lactate dehydrogenase values both in vitamin B_{12} and folic acid deficiency anaemias [4 9]

In azotemia the glucose-6-phosphate dehydrogenase activity of red cells has been found to be increased [3 6 24] This has been attributed to increased erythropoiesis or reduced mean age of the cells. In the present study the TKA of red cells remained largely within the normal variation range, and did not differ statistically significantly from the controls. Figure 1 shows, however that 3 of the 13 patients had TKA values distinctly higher than the control subjects.

The thyroid hormone is known to stimulate the oxygen consumption of the red cells. This especially seemed to be the case in hexose monophosphate shunt [17] As a possible result it has been found that the glucose-6-phosphate dehydrogenase activity of the red cells is increased [18 21 27] in thyrotoxic patients. A similar result has been obtained on healthy subjects after administration of thyroxin [20] It might be expected that activities of several enzymes of hexose monophosphate shunt should be increased. Figure 4 shows, however that the TKA of red cells is largely within the range of normal variation in about 20% the TKA was even below the normal range. In one patient, a man of 60 the TKA was only 31 units. Simultaneously he had a very violent hyperthyroidism and peripheral neurites in the lower limbs. It is quite possible, theoretically that the need of cocarboxylase is increased in hyperthyroidism while its deficiency might lead to reduced TKA values. The values of six patients with hypothyroidism must be considered completely normal.

A few cases of the group of 'Various Haematological Conditions' (table IV) deserve attention. Case No. 6 with polycythaemia vera

Acta haemat. 39: 333–344 (1968)

I. Medizinische Universitätsklinik Wien (Vorstand: Prof. Dr. E. Deutsch)

Untersuchungen über Erythrokinetik und Erythrozytenstoffwechsel bei Thalassaemia minor[1]

N. Honetz, K. Moser, E. Neumann und H. Seipel

Während die homozygote Form der Thalassämie eine meist zum Tode führende, schwere Erkrankung darstellt, weist die heterozygote Form nicht nur eine ausserordentliche Variabilität, sondern auch einen sehr unterschiedlichen Intensitätsgrad der Symptomatik auf, deren Ursache bis heute noch nicht geklärt erscheint. Es ist daher verständlich, dass Versuche unternommen wurden, weitere Unterteilungen nach verschiedenen Gesichtspunkten vorzunehmen [10, 34]. Dabei hat sich die Einteilung der heterozygoten Form der Thalassämie in jene Fälle, die lediglich am Vorliegen von Thalassämiemerkmalen, wie Facies microcythaemica, Mikrozytose mit vermehrter osmotischer Resistenz, Hämoglobin-A_2-Vermehrung zu erkennen sind (Thalassaemia minima oder Thalassaemia trait) und solche Fälle, die mit Krankheitserscheinungen einhergehen (Thalassaemia minor), allgemein durchgesetzt. Nur bei einem Teil dieser heterozygoten Formen lassen sich Zeichen eines vermehrten Erythrozytenabbaues feststellen, für den die verstärkte mechanische Zerbrechlichkeit der Erythrozyten verantwortlich gemacht wird. Die Ursache derselben ist allerdings bis heute nicht bekannt und wird mit der defekten Erythropoese in Zusammenhang gebracht [14]. Zur weiteren Klärung der Pathogenese wurden daher in letzter Zeit Stoffwechseluntersuchungen an Erythrozyten Thalassämiekranker durchgeführt. So konnten Gaionani *et al.* [18] bei drei Fällen von Thalassaemia major eine deutliche Aktivität der Glyzerophosphatdehydrogenase-Aktivität in den Erythrozyten nachweisen, während bei Gesunden und bei an Thalassaemia minor Erkrankten die Aktivität dieses Fermentes in den Erythrozyten

[1] Herrn Prof. Dr. F. Reimann zum 60. Geburtstag gewidmet.

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nicht vorhanden war. Eine Aktivitätszunahme fast aller Enzyme in den Erythrozyten mit Ausnahme der Phosphofruktokinase, die bei Thalassämie vermindert ist, fanden BELFIORE *et al.* [5]. Weiter konnten ZÜRCHER *et al.* [41] wechselnde Befunde über den Nukleotidgehalt der Erythrozyten und CORRADI *et al.* [11] eine Verminderung der Adeninnukleotide in den Erythrozyten thalassämischer Kinder nachweisen.

Wir hatten nun bei einer Patientin mit Thalassaemia minor, bei der mittels ^{51}Cr und ^{59}Fe Aufschluss über Ort und Ausmass der Erythrozytenbildung und des Erythrozytenabbaues erhalten werden konnte, sowie bei deren Tochter, die Thalassämiemerkmale ohne Krankheitssymptome aufwies, Gelegenheit, Untersuchungen des Erythrozytenstoffwechsels durchzuführen.

Kasuistik

Pat. T. D., eine aus Serbien gebürtige, 34 Jahre alte Fremdarbeiterin wurde mit Hepatitisverdacht an die Klinik gewiesen. Anamnestisch wird das Vorkommen von Gelbsucht und Bluterkrankungen in der Familie verneint. Nach einem fieberhaften Infekt 2 Monate vor der Klinikaufnahme wurde ein Sklerenikterus bemerkt, der in der Folgezeit immer wieder auftrat. Die Pat. fühlt sich matt, schwindlig und appetitlos. Status: Mit Ausnahme eines den Rippenbogen um 3 QF überragenden, derben Milztumors und der blaßikterischen Hautfarbe keine Besonderheiten. Schädelform unauffällig, Leber nicht vergrößert. Laborbefunde: Blutsenkung n. W. 2/4 mm, WAR negativ, Serumlabilitätsproben, Eiweißwerte und Elektrophorese unauffällig, SGOT 16 mE, LAP 9,7 mE, LDH 133 mE, Gesamtbilirubin 2,9 mg%, davon 2,3 mg% indirektes Bilirubin, Serumeisen 80 γ%. Bromsulphthaleinretention nach 45 min normal, Harnbefund chemisch und morphologisch unauffällig, Cholecystographie nach peroraler Füllung o. B., Röntgenaufnahmen des Skelettes mit Ausnahme einer grobporigen Struktur der Schädeldecke ohne Besonderheiten. Hämatologische Befunde: Ery 4,46 Mill., Hb 8,9 g%, Hk 23%, FI 0,62, Retikulozyten 18‰, Hb_E 20 γγ, Vol_E 64 μm^3, Hb-Index 29%, Leuko 4500 bei unauffälliger Verteilung, Thrombozyten 220000.

Vermehrte Aniso- und Poikilozytose, basophile Tüpfelung und Targetzellen in einem höheren Prozentsatz. Osmotische Resistenz deutlich verbreitert. Im Alkalidenaturierungstest nach SINGER und Mitarb. 3,5% Hb-F. $Hb\text{-}A_2$ war in einer Menge von 5,2% vorhanden*. Direkter Coombs-Test, Blockingtest und indirekter Coombs-Test negativ. Kälteagglutinintiter 1:8, A- bzw. Hypohaptoglobinämie, Säurehämolysetest negativ. Sternalmark: 80% Zellen der erythropoetischen Reihe, davon 85% Sideroblasten, Siderozyten 3%. Zahlreiche basophil getüpfelte Normoblasten. Granulopoese unauffällig.

Pat. M. S., 16 Jahre, Tochter der Pat. T. D. Die Anamnese ist unauffällig und die Pat. beschwerdefrei. Kein Milztumor, kein Ikterus und keine auffällige Schädelform. Keine sicheren Zeichen für eine gesteigerte Hämolyse. Serumbilirubin und Serumeisen im Normbereich, Haptoglobine nicht vermindert. Ery 5,97 Mill., Hb 11,0 g%, Hk 37%, FI 0,63, Hb_E 18,4 γγ, Vol_E 62 μm^3, Hb-Index 34%, Retikulozyten 14‰

* Wir danken Herrn Dozent KLEIHAUER, Kinderklinik München, für die quantitative $Hb\text{-}A_2$-Bestimmung.

keine basophile Tüpfelung der Erythrozyten. In der Stärkegelelektrophorese zeigte sich eine Vermehrung von $Hb\text{-}A_2$, allerdings wurde eine quantitative Bestimmung nicht durchgeführt.

Methodik

1. Erythrokinetik. Die Untersuchungen mit ^{51}Cr und ^{59}Fe erfolgten nacheinander so, dass das mit ^{59}Fe markierte Plasma erst unmittelbar nach Abschluss der Chromoberflächenaktivitätsmessungen injiziert wurde. Die Messungen wurden mittels eines Szintillationszählers durchgeführt, wobei zur Trennung der Energiebereiche lediglich für die Blutmessungen ein Einkanalanalysator zur Verfügung stand. Bei den Eisenoberflächenaktivitätsmessungen wurde die energieärmere Chromstrahlung durch entsprechende Einstellung des Diskriminators ausgeschaltet. Die Markierung von autologem Patientenplasma und autologen Patientenerythrozyten erfolgte in vitro mit etwa 20 µC ^{59}Fe-Chlorid bzw. mit 100 µC Na-Chromat (^{51}Cr). Bestimmt wurden Erythrozytenvolumen, Plasmaeisenclearance, Eisenutilisation und scheinbare Chromüberlebenszeit der Erythrozyten. Außerdem wurden Oberflächenaktivitätsmessungen über Leber, Milz, Herz und Sacrum durchgeführt.

2. Stoffwechseluntersuchungen in den Erythrozyten. a) Enzymaktivitätsbestimmungen. Zitratblut wird 3mal in isotoner NaCl-Lösung gewaschen. Die Erythrozyten werden in Phosphat-Ringer-Lösung (pH 7,4) suspendiert und auf 2,5 bis 3 Mill. Erythrozyten/µl eingestellt. Ein ml der Suspension wird mit 1 ml Aqua bidest., 0,7 ml m/20 Triäthanolaminpuffer (pH 7,5) und 0,3 ml gesättigter und filtrierter Digitoninlösung versetzt, 15 min im Kühlschrank stehengelassen und anschließend 15 min bei 350 U/min zentrifugiert und dekantiert. Alle Enzymbestimmungen wurden bei pH 7,5 und +25 C in einer durchströmten Testküvette bei einer Wellenlänge von 366 nm im Photometer »Eppendorf« durchgeführt. Die Fermentaktivität wurde nach Bücher durch Bestimmung der Laufzeit der Reaktion nach einem Vorlauf zwischen 2 Extinktionsmarken gemessen. Die Angabe der Enzymaktivität erfolgt in internationalen Einheiten (IU). 1 IU wird als die Enzymmenge definiert, die 1 Mikromol Substrat/Zeiteinheit umsetzt.

Die Bestimmung der Hexokinase erfolgte nach Gerlach und Löhr [19], die der Hexosephosphatisomerase nach Slein [38], der Fruktose-6-Phosphatkinase nach Ling [27], der Diphosphofruktose-Aldolase nach Bücher [8], der Triosephosphatisomerase nach Beisenherz [4], der Glyzerinaldehyd-3-phosphat-dehydrogenase nach Delbrück und Bücher [15], der 3-Phosphoglycerat-*1*-kinase und der Phosphoglyceromutase nach Bücher [6], der Enolase nach Bücher [7], der Pyruvatkinase nach Bücher und Pfleiderer [9], der Laktatdehydrogenase nach Kubowitz und Ott [26], der Malatdehydrogenase nach Ziez [15], der Glukose-6-Phosphatdehydrogenase nach Kornberg und Horecker [25], der 6-Phosphoglukonat-Dehydrogenase nach Horecker und Smyrniotis [23], der Glutathionreduktase nach Racker [33], der Myokinase nach Adam und Bücher [2], der Mg^{++}-aktivierten ATP-ase nach Löhr und Gerlach [28].

b) Substratbestimmungen. Zur Bestimmung von Adenosintriphosphat (ATP), Adenosindiphosphat (ADP) und Adenosinmonophosphat (AMP) wurde Vollblut entnommen und sofort in eisgekühlte 6%ige Perchlorsäure eingebracht. Der enteiweißte Niederschlag wurde abzentrifugiert, anschließend erfolgt die Neutralisierung mit 1-n KOH und Abtrennung des Kaliumperchlorates. ATP, ADP und AMP wurden enzymatisch nach Adam [1] bestimmt. Die enzymatische Laktatbestimmung erfolgte nach Hohorst [22] und die Pyruvatbestimmung nach Czok und Bücher [13]. Das reduzierte Glutathion wurde nach der Methode von Grunert und Phillips [20], die Glutathionstabilität nach Beutler [6] bestimmt. Der Kalium- und Natriumgehalt der Erythrozyten wurde mit dem Flammenphotometer gemessen.

c) Manometrische Bestimmungen. Zur Erfassung des Gesamtstoffwechsels der Erythrozyten wurde die Sauerstoffaufnahme und die Kohlendioxydbildung nach War-

[illegible] [40] mit der Gefäßpaarmethode bestimmt. Gleichzeitig wurde auch die Laktat und Pyruvatbildung pro Stunde gemessen. Dazu wurden die Erythrozyten in der vorher beschriebenen Weise gewaschen und in Bicarbonat Ringer-Lösung mit Glukosezusatz suspendiert. Die Konzentration der Erythrozyten in dieser Suspension betrug 3 Mill./µl. Hämoglobin und Methämoglobinbestimmungen wurden nach dem Prinzip der Kohlenoxydhämoglobinmethode von Kruse [24] durchgeführt.

Ergebnisse

Erythrokinetik. Es findet sich bei Pat. T D eine deutlich beschleunigte Plasmaeisenclearance von 23 min, woraus sich bei einem Eisenspiegel von 80 µg% ein mit 2 4 mg/100 ml Blut die auf etwa das Vierfache der Norm erhöhter Plasmaeisenturnoverwert ergibt. Im Gegensatz zu diesem erhöhten Plasmaeisenturnoverwert ist jedoch die Eisenutilisation mit nur 35 6% sehr niedrig (Abb. 1) Wie in der Abbildung ersichtlich, erfolgt der Eisenanstieg über dem Sacrum rasch und hat bereits nach 4 Stunden seinen maximalen Wert erreicht, der allerdings infolge der starken Ausdehnung des blutbildenden Markraumes nicht hoch ist. Der Aktivitätsabfall geht nur langsam vor sich, ohne dass nach 13 Tagen der Ausgangswert erreicht wurde. Die Leberaktivität nähert sich nach anfänglichem Abfall etwa am 10. Tag wieder dem Ausgangswert, während die Milzaktivität nach vorübergehendem Abfall den Anfangswert bald übersteigt. Bis zum 3. Tag nimmt die Aktivität hier rasch und darnach nur mehr langsam zu. Die Untersuchungen mit ^{51}Cr ergeben eine Verkürzung der scheinbaren halben Erythrozyten lebensdauer auf 16 Tage, ohne dass sich eine nennenswerte Aktivitätszunahme über einem der gemessenen Organe feststellen lässt.

Erythrozytenstoffwechseluntersuchungen. Aus der Tab. I ist zu erkennen, dass von den Erythrozytenenzymen bei Pat. T und ihrer Tochter M. eine deutlich nachweisbare Glyzerophosphatdehydrogenase-Aktivität in den Erythrozyten vorhanden ist. Es fällt weiter die deutlich verminderte Mg^{++} abhängige ATP-ase Aktivität in den *Erythrozyten beider Patientinnen auf. Von den übrigen* Enzymaktivitäten sind die Di Phosphofruktose Aldolase, die Pyruvatkinase und Glukose-6-Phosphatdehydrogenase-Aktivität in den roten Blutzellen beider Patientinnen etwas erhöht, die Fruktose-6-Phosphatkinase und die Laktatdehydrogenase Aktivität sind bei der Pat. T in den Erythrozyten erhöht, während bei ihrer Tochter keine Vermehrung der Aktivität gefunden wurde. Die Enolase-Aktivität ist bei beiden Patientinnen in den roten Blutzellen etwas vermindert. Bei der Tochter von Pat. T ist die Hexokinase und die

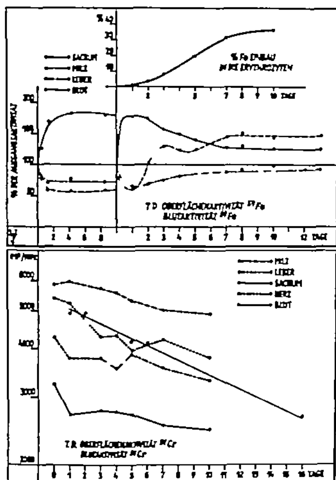


Abb. 1

Malatdehydrogenase Aktivität in den Erythrozyten geringfügig erhöht. Die Substratuntersuchungen (Tab. II) zeigten, daß der ATP und ADP-Gehalt der Erythrozyten bei Pat. T deutlich vermindert ist, der AMP-Gehalt ist unauffällig Auch die Tochter M. weist in den Erythrozyten einen verminderten ATP-Gehalt auf, ADP und AMP sind unauffällig Bei Pat. T ist das reduzierte Glutathion in den Erythrozyten etwas vermindert, das Methämoglobin leicht erhöht. Die Untersuchung des Natrium- und Kalium gehaltes der Erythrozyten ergab bei beiden Patientinnen eine Verminderung des Kaliumgehaltes, während der Natriumgehalt

Tabelle I Normalwerte der Enzymaktivitäten mit Streubreiten in IU/10^{10} Erythrozyten und Werten von Patientin T D. und M. S.

	Normalwerte	±	Pat. T	Pat. M.
Hexokinase	1,84	0,98	1,63	3,84
Glukose-P Isomerase	83 0	8,0	96,6	90,0
Fruktose-6-P-Kinase	15,0	2,5	19 1	17,2
Di-P-Fruktose-Aldolase	5,5	0,5	10,6	10,5
Triose P Isomerase	628,0	89,0	581,0	629,0
Glycerophosphat DHG			4,8	4,9
Glycerinaldehyd-3-P-DHG	225,0	34,2	217,0	273,0
3-P-Glycerat-1 Kinase	303,0	56,5	334,0	323,0
P-Glycerat Mutase	72,5	10,3	66,5	68,9
Enolase	28,5	3,4	20,7	18,3
Pyruvatkinase	40,7	6,1	39,1	53,6
Laktatdehydrogenase	283,0	41,5	381,0	232,0
Glukose-6-P DHG	13,9	1,54	20,4	17,3
6-P-Glukonat DHG	5,7	1,2	6,45	7 4
Glutathionreduktase	6,1	2,1	8,45	8,05
Malatdehydrogenase	244,0	36,2	180,0	359,0
Mg^{++}-abhängige ATP-ase	33,0	3,7	5,9	11,3

Tabelle II. Stoffwechselleistungen der Erythrozyten, Substrate in Mikromol/10^{10} Erythrozyten (37° C, pH 7 4 Bikarbonat Ringer Lösung 0,01 M Glukose) Glutathionstabilität (Abfall) % und Methämoglobingehalt in % sowie Kalium und Natriumgehalt der Erythrozyten von Gesunden und Patientin T und M.

	Normalwert	±	Pat. T	Pat. M.
O_2-Aufnahme/h	4,20	0 4	2,85	
CO_2-Bildung/h	7,68	1,0	11,3	
Pyruvatbildung/h	0,9	0,3	0,69	
Laktatbildung/h	28,2	8,6	38,50	
Glukoseverbrauch/h	12,1		25,25	
ATP	20,8	1,8	8,13	15,2
ADP	2,2	0,2	0,69	1 14
AMP	1 1	0,4	1,22	0,7
Reduziertes Glutathion (GSH)	20,0	3,9	15,7	16,0
GSH-Stabilität (Abfall) %	bis 23%		9,3	11,5
Methämoglobin (%)	1,0		1,8	0,3
K (mval/l)	92,0	4 7	78,0	85,4
N (mval/l)	19,0	4,2	29,0	26,3

vermehrt war Die Untersuchung der Stoffwechselleistungen der Erythrozyten von Pat. T zeigte dass die Erythrocyten einen doppelt so hohen Glukoseverbrauch und eine erhöhte Laktatbildung auf

weisen. Die Pyruvatbildung ist unauffällig. Die Sauerstoffaufnahme ist geringfügig vermindert, die Kohlendioxydbildung etwas erhöht.

Diskussion

Bei der Untersuchung der Erythrozytenenzyme unserer beiden Patientinnen fällt die nachweisbare Aktivität der Glyzerophosphatdehydrogenase auf, die normalerweise, wie LÖHR und WALLER [29] zeigen konnten, in den Normozyten fehlt. Die Autoren konnten bei 3 Kranken mit Thalassaemia major eine erhöhte Aktivität dieses Enzymes in den Erythrozyten nachweisen, bei einem Fall von Thalassaemia minor war die Aktivität der Glyzerophosphatdehydrogenase nicht vorhanden. Die Glyzerophosphatdehydrogenase, die im Nebenweg der Glykolyse wirksam ist, benötigt für die Reaktion mit dem Substrat Dihydroxyazetonphosphat NADH, als Co-ferment (Abb. 2). Es konkurriert die Glyzerophosphatdehydrogenase in den Erythrozyten bei der Thalassaemia minor mit der Laktatdehydrogenase und der Methämoglobinreduktase um das bei der oxydierenden Gärungsreaktion gebildete $NADH_2$. Damit könnte der leicht erhöhte Methämoglobingehalt der Erythrozyten unserer Patientin erklärt werden. Dass die Laktatbildung, wie erwartet, nicht vermindert, sondern eher leicht erhöht gefunden wurde, könnte mit dem hohen Glukoseverbrauch der Erythrozyten erklärt werden. Eine verminderte ATP-ase-Aktivität in den Erythrozyten ist bisher erst von HARVALD *et al.* [21] bei 3 Fällen von nicht sphärozytärer hämolytischer Anämie beschrieben worden. Bei der Thalassaemia minor ist bisher eine Aktivitätsverminderung dieses Enzymes nicht gefunden worden. Die Bedeutung der Mg^{++} ATP-ase für den aktiven Transport von Kalium und Natrium durch die Zellmembran ist bekannt [32, 36, 37]. Wahrscheinlich ist die Elektrolytstoffwechselstörung der Erythrozyten und damit auch die vermehrte Hämolyse damit in Verbindung zu bringen.

Der verminderte ATP-Gehalt, der verminderte bzw. unauffällige ADP- und AMP-Gehalt der Erythrozyten *bei uneingeschränkter* Pyruvat- und Laktatbildung sprechen eher für eine Nukleotidbildungsstörung bei uneingeschränkter Glykolyse. Auch die Verminderung des Nukleotidgehaltes der Erythrozyten könnte für die Elektrolytstoffwechselstörung und verstärkte Hämolyse verantwortlich gemacht werden. *Die von uns teilweise erhöht gefundenen* Enzymaktivitäten in den Erythrozyten weisen auf eine jüngere Zellpopulation hin, wie es im Rahmen der Hämolyse erwartet wird.

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An Unusual Hematological Syndrome with Pyruvate-Kinase Deficiency and Thalassemia minor in the Kindreds

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Hereditary hemolytic anemia secondary to erythrocyte pyruvate kinase deficiency is now a well established clinical entity. Several examples of this disorder have been observed in our laboratory in addition to the original 7 cases [1, 2], and there have been numerous reports from other laboratories [3–10]. From these reports a fairly consistent clinical pattern has emerged which characteristically includes three prominent features. First, there is a chronic hemolytic anemia which usually responds minimally to splenectomy. Second, erythrocyte morphological changes are usually nondescript with only slight macrocytosis and occasional irregularly contracted cells. And, finally, erythrocyte autohemolysis is enhanced by sterile incubation and usually not corrected by the addition of glucose.

Recently, we have observed a patient with a hemolytic syndrome who exhibited at least a partial deficiency of erythrocyte pyruvate kinase. There were, however, many atypical features, including apparent major benefit from splenectomy, prominent erythrocyte shape changes with many cells resembling acanthocytes, and a pattern of autohemolysis distinctly different from that usually *noted in pyruvate kinase deficiency*. Although numerous authors have commented upon erythrocyte morphologic abnormalities in pyruvate kinase deficiency, such changes have generally

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not been very prominent. A notable exception is the case reported by Oski et al [11] Because of the clinical pattern, it was suspected that a second hematologic abnormality in addition to pyruvate kinase deficiency might be present. To investigate this possibility family studies were performed and β-thalassemia (B thalassemia) minor in addition to pyruvate kinase deficiency was found in the kindreds. The hematologic, biochemical and genetic studies of this patient and his family constitute the subject of this report.

Materials and Methods

Routine hematologic studies were performed by standard methods. The autohemolysis test was done on defibrinated blood as previously described. Osmotic fragility was tested by the method outlined by Dacie [12]. The activities of erythrocyte hexokinase, glucosephosphate isomerase, phosphofructokinase, fructosediphosphate aldolase, glyceraldehydephosphate dehydrogenase, triosephosphate isomerase phosphoglycerate kinase, phosphoglyceromutase phosphopyruvate hydratase pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, and glutathione reductase were assayed as previously noted [7 13–15].

Glutathione stability was performed essentially as described by Beutler [16] with quantitation of the reduced glutathione by a slight modification of the 5,5 dithiobis (2-nitrobenzoic acid) method of Beutler, Duron and Kelly [17].

Heinz body induction was quantitated as described by Beutler, Dern and Alving [18].

Vertical descending starch block electrophoresis was done according to the method by Smithies [19] using a phosphate buffer system at pH 7.0 according to the method of Gammack, Huisman, Shooter and Gerald [20]

Lactate production from several different glycolytic intermediate compounds was evaluated by the addition of the separate intermediates to erythrocyte hemolysate Requisite co-factors were added when needed. The lactate generated after period of incubation was quantitated enzymatically [21] with lactate dehydrogenase

Hemoglobin A_2 was determined electrophoretically on cellulose acetate Fetal hemoglobin was determined by the alkali denaturation method of Singer, Chernoff and Singer [22].

Case Report

The patient, S. R., a caucasian male student of partial Italian ancestry was first studied in our laboratory at 17 years of age. No abnormalities were noted at birth and he remained in excellent health throughout childhood. At 14 years of age a tonsillectomy was performed because of repeated episodes of pharyngitis. At this time he was found to be anemic with a hemoglobin concentration of 10.5 g/100 ml. At age 18, during an acute febrile illness, he was seen by a physician who noted splenomegaly Except for prominent frontal bossing, the physical examination was otherwise unremarkable Hemoglobin concentration varied from 8.1 to 10.5 g/100 ml, and reticulocyte count was 2.8%. Peripheral blood many acanthocyte-like erythrocytes there was normoblastic erythroid hyperplasia of the bone marrow Because of the history of abdominal trauma during a

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We thank Drs. William R. Bergren and Norma Shore, Children's Hospital of Los Angeles, Cal., for obtaining these procedures.

football game four weeks earlier splenectomy was performed because of the possibility of splenic hematoma. The spleen was enlarged to four times normal size; there was no evidence of hematoma. Microscopically: Chronic congestion with moderate amount of hemosiderin deposition thickening of the cords and rarefaction of the white pulp.

Following splenectomy the hemoglobin concentration increased and varied from 13.6 to 15.0 g/100 ml. The reticulocyte count varied from 0.7 to 1.1%. The red cell indices were consistent with slight macrocytosis. Examination of the Wright-stained smear of the peripheral blood (fig. 1) showed persistence of the morphologic abnormalities present prior to splenectomy with approximately 75% of the erythrocytes showing contraction with numerous irregular blunted projections similar to acanthocytes. When erythrocyte shape was fixed by adding whole blood to formalized saline immediately after withdrawal from the antecubital vein, according to modification of the Srlama Test [23], 42% of the patient's erythrocytes and less than 1% of the normal control erythrocytes showed abnormal spiculation. If, however, the erythrocyte shape fixation were not performed for 15 min or 2 h after withdrawal from the vein, the percentage of observed spiculation sharply increased in the patient whereas there was no increase in the normal blood. It is clear that the shape abnormality observed in the patient was present immediately after the blood was removed from the vein and increased rapidly thereafter. In addition to the marked anisopoikilocytosis, there was marked macrocytosis, slight microspherocytosis, and occasional ovalocytes, target cells and tailed poikilocytes. The cells were essentially normochromic with minimal polychromatophilia rare basophilic stippling and numerous Howell-Jolly bodies present. The leukocyte count was 10,900/mm³ with 2 neutrophilic bands, 38 segmented neutrophils, 1 basophil, 3 eosinophils, 42 small lymphocytes, and 14 monocytes. The platelet count was 698,000/mm³. Hemoglobin electrophoresis showed an AA pattern, and the A_2 fraction was 3.5%. The fetal hemoglobin was 2.2%. Starch block electrophoresis using phosphate buffer 0.054 M, pH 7.0, showed no migration toward the anode such as seen with hemoglobin H or Bart's hemoglobin. No inclusion bodies were noted on incubation with brilliant cresyl blue. Direct and indirect Coombs tests were negative; the serum haptoglobin was 88 mg% and the total bilirubin was 0.33 mg% with direct value of 0.09 mg%. Serum iron was 129 mg% and total iron-binding capacity was 388 mg% with 33% saturation. Serum protein electrophoresis revealed no abnormalities, the cholesterol was 178 mg% and the alkaline phosphatase was 4.8 Shinowara Units (Normal 2–9 Units). The serum beta-lipoprotein was found to be within normal limits. Red cell stromal lipid analysis (total lipid, lipid phosphorus, cholesterol, phospholipid fractionation, and the pattern of phospholipid fatty acids) was found to be within normal limits[1].

Results

All the erythrocyte glycolytic enzymes mentioned under Materials and Methods were assayed on the patient, parents, siblings, maternal grandparents, paternal grandmother and paternal aunt. Pyruvate kinase activity was found to be deficient and the results are shown in table I. However the degree of deficiency in the propositus was less marked than that usually observed in homozygous pyruvate kinase deficiency [2].

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[1] We thank Dr. Robert C. Neerhout, Department of Pediatrics, UCLA for doing these assays.

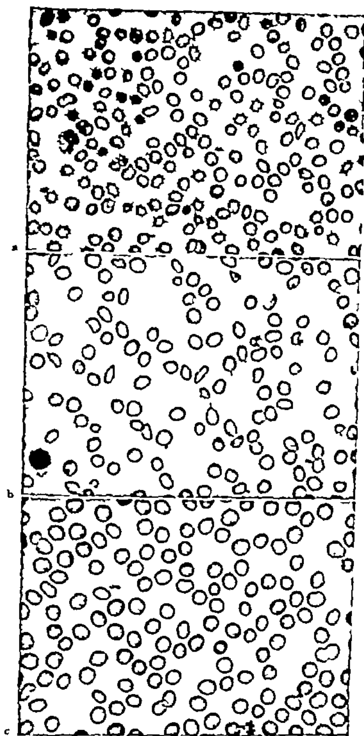


Fig 1 Photomicrographs of blood smear from the patient nd his parents. ( ) Propositus, (b) Father ( ) Mother

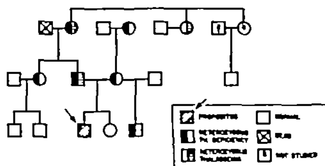


Fig. 2. Graphic representation of family studies.

Table I. Pyruvate kinase activity of erythrocytes and leukocytes of the patient and his family members

Subject	*Enzyme Activity* (µmoles of substrate utilized/min/10^{10} cells) *Erythrocytes*	*Leucocytes*
Normal range	2.0–3.4	944–1436
Patient (3 determinations)	0.76	1745
Father	1.83	1118
Mother	1.48	1118
Sister	2.83	1444
Half-brother	1.03	1303
Maternal Grandfather	2.24	1431
Maternal Grandmother	1.30	1444
Paternal Grandmother	1.09	1475
Paternal aunt	0.77	1593
Paternal first cousin	1.94	1133
Paternal great aunt	3.17	
Paternal first cousin	2.46	

Data previously published. Erythrocyte pyruvate kinase activities below 1.73 units are characteristic of heterozygous enzyme deficiency.

Family Studies

The results of family studies are summarized graphically in figure 2. Parents. The *father* was of Italian ancestry. Physical examination revealed splenomegaly. Anemia was present with a hemoglobin concentration of 10.6 g/100 ml and the volume of packed cells was 37%. Red cell count was relatively increased to 5.69 million/mm³. The mean corpuscular volume was 65 µm³, the mean corpuscular hemoglobin was 18.6 pg and the mean corpuscular hemoglobin

Table II. Autohemolysis studies of the red blood cells of the patient and his family members

Additive final concentration (M)	None	Glucose	Adenosine	Adenosine triphosphate
	–	0.26	0.02	0.02
Normal mean % autohemolysis	1.7	0.2	0.2	0.2
Patient	10.6	0.8	0.1	0.2
Father	2.4	0.4	0.6	0.6
Mother	1.0	0.0	0.0	0.8
Sister	1.2	0.1	0.1	0.3
Half-brother	0.7	0.1	0.1	0.8

city were noted and linearized on a Lineweaver-Burk plot with the Michaelis-Menten constant calculated from the point of intersection with the abscissa at $1/K_m$. No difference was noted in the K_m value for the patient's erythrocytes and leukocytes as compared with the normal controls.

Other Studies

Autohemolysis (table II). There was marked autohemolysis of the patient's erythrocytes with almost complete correction by glucose, adenosine and adenosine triphosphate. These findings are in contrast to those usually noted in pyruvate kinase deficiency hemolytic anemia in which marked autohemolysis is not corrected by glucose or adenosine, but only by adenosine triphosphate. The pattern of autohemolysis observed in this patient is similar to that observed in hereditary spherocytosis, but the lack of characteristic findings in either parent, the absence of prominent spherocytosis, and the osmotic fragility studies all rule strongly against this entity as a diagnostic consideration. The pattern of autohemolysis in diverse hemolytic states is of interest, however. It seems quite clear that characteristic differences can be correlated with the various sites of metabolic impairment when they are known. Thus, chronic hemolytic anemia with hexokinase deficiency [15] and usually with glucose-6-phosphate dehydrogenase deficiency are associated with a very slight increase in autohemolysis which is partially corrected by glucose. Classic pyruvate kinase deficiency hemolytic anemia is characterized by marked autohemolysis usually uncorrected by glucose, and triosephosphate isomerase hemolytic anemia [14] by marked autohemolysis with complete correction by glucose. The finding of a deviant pattern of autohemolysis in our patient thus

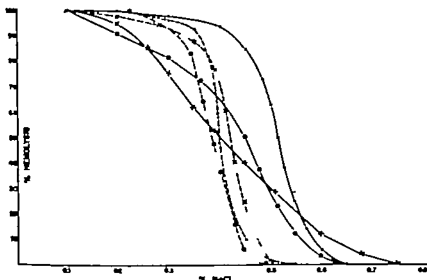


Fig. 3. Erythrocyte osmotic fragility of the patient and his mother

Normal control	fresh defibrinated blood	●———●
	24 h incubation	●– – –●
Patient:	fresh defibrinated blood	———x
	24 h incubation	–
Patient's mother:	fresh defibrinated blood	o———o
	24 h incubation	o – o

suggests a variation in the mechanism of impairment of erythrocyte viability. The nature of these altered mechanisms remains obscure. Slight autohemolysis was noted in the erythrocytes of the father and paternal aunt and was correctible by additives of glucose, adenosine and adenosine triphosphate. Autohemolysis was within normal limits in all other family members studied.

Erythrocyte osmotic fragility (fig. 3 and 4). Osmotic fragility was quantitated on erythrocytes from the patient and his immediate family members, as well as on red cells from several of the paternal relatives. There was a clear increase in resistance to osmotic lysis in red cells from the father and from other family members with thalassemia. A marked further increase in osmotic resistance was noted after sterile incubation (fig. 4). We have observed similar alterations on several occasions with erythrocytes from patients with B thalassemia minor. Erythrocytes from the mother (fig. 3) and the patient's siblings failed to reveal any abnormalities in

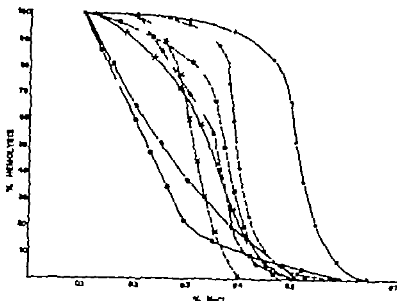


Fig. 4. Erythrocyte osmotic fragility of family members with thalassemia: the father, paternal grandmother and paternal great aunt.

Normal controls	fresh defibrinated blood	●- - - - - - ●
	24 h incubation	●———————●
Father	fresh defibrinated blood	○- - - - - ○
	24 h incubation	○———————○
Paternal grandmother	fresh defibrinated blood	○○- - -○○
	24 h incubation	○○———————○○
Paternal great aunt	fresh defibrinated blood	x- -x
	24 h incubation	x———————x

osmotic fragility when studies were performed on fresh defibrinated blood. After sterile incubation, however, a slight tail of osmotically resistant red cells appeared in the curves obtained from each of these individuals. A more marked degree of flattening of the curve was noted in studies of incubated erythrocytes from the patient with findings somewhat intermediate between those of the parents.

Discussion

The data demonstrated the presence of both heterozygous pyruvate kinase deficiency and thalassemia in the kindreds studied. The patient's mother and her family members exhibited heterozygous pyruvate kinase deficiency only, the father and several of his family members were shown to have both heterozygous pyruvate kinase deficiency and heterozygous beta thalassemia.

Heterozygous pyruvate kinase deficiency has uniformly been found in both parents in all cases of homozygous pyruvate kinase deficiency hemolytic anemia when the parents were available for study. Thus the presence of heterozygous enzyme deficiency in both parents suggests that our patient may be affected by the homozygous form of the enzyme deficiency. In our laboratory, however, erythrocyte pyruvate kinase activity has generally been much lower in patients homozygous for the enzyme deficiency. Activity has varied in our experience from 0.00–0.83 units, but the higher values have been observed only in subjects in whom recently transfused normal erythrocytes were present. Enzyme activity in heterozygous family members has varied from 0.63–1.73 units [25]. It is thus apparent that our patient with an erythrocyte enzyme activity averaging 0.76 units falls into an indeterminate range consistent with either the heterozygous or homozygous form of the deficiency state. It should be emphasized that there is no quantitative relationship between the degree of enzyme deficiency and the severity of the clinical findings in patients with homozygous pyruvate kinase deficiency. The principal clinical application of enzyme activity quantitation is to establish the genetic status of the individual. In our patient it was not possible to clearly resolve the question of heterozygosity or homozygosity by this criterion.

In previously observed instances of indeterminate enzyme levels, both clinical and genealogic characteristics have generally permitted conclusions regarding the patient's genetic status. Since apparently only persons with the homozygous form of pyruvate kinase deficiency exhibit signs of impairment of erythrocyte viability, the history of hemolytic anemia prior to splenectomy is itself suggestive of the homozygous state. It should be emphasized, however, that patients with typical homozygous deficiency generally exhibit only minor improvement after splenectomy. BOWMAN's patients [5] are a conspicuous exception, and some others have exhibited modest improvement.

One of the most striking features of this case was the prominence of erythrocyte shape changes, especially the many acanthocyte-like forms. Somewhat similar changes have been observed in some cases of liver disease, uremia and various malignant tumors. In addition, acanthocytes are uniformly found in congenital beta-lipoprotein deficiency. There were no findings consistent with any of these conditions in our patient. Variable numbers of irregularly contracted

red cells have been noted previously in pyruvate kinase deficiency hemolytic anemia but, except for one case with a very severe hemolytic anemia [11] these have never been as striking as in this patient. It appears unlikely that the observed morphologic changes simply represent an exaggeration of those commonly seen.

In a recent case reported by HSU, ROBINSON and ZUELZER [26] a family was found in which certain members exhibited very low pyruvate kinase activity but only extremely mild hematological abnormalities. This was thought to result from double heterozygosity from two interacting genes associated with pyruvate kinase deficiency. However no substantial erythrocyte morphological abnormalities similar to those in our case were described. The existence of a variant of pyruvate kinase is, of course, a possibility that cannot be ruled out.

The paternal kindred exhibited evidence of heterozygous thalassemia in addition to heterozygous pyruvate kinase deficiency. In addition to anemia, microcytosis, poikilocytosis and relative erythrocytosis, elevation of A_2 hemoglobin was observed in affected family members, consistent with beta-thalassemia.

It would be attractive to hypothesize that the unusual hematologic syndrome manifested by this case is the result of combined inheritance of both pyruvate kinase deficiency and beta thalassemia. While it has been established clearly that the heterozygous form of both of these disorders is present in the patient's kindred, it has not been possible to define inheritance of beta thalassemia in the patient himself. Just as there is some uncertainty as to the patient's genetic status with regard to pyruvate kinase deficiency there is no direct evidence that he has inherited the thalassemic trait despite its presence in the paternal kindreds. The patient had no elevation of A_2 hemoglobin, even though such elevations were uniformly noted in paternal family members with thalassemia, and there was no microcytosis or relative erythrocytosis. Also, it is somewhat difficult to conceptualize the mechanisms of interreaction between a trait affecting hemoglobin synthesis and another concerned with energy metabolism.

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Summary

An unusual hemolytic syndrome with pyruvate kinase deficiency was observed in patient of partial Italian ancestry. Erythrocyte shape changes were prominent both before and after splenectomy with substantial improvement following the latter. Autohemolysis was corrected by glucose and adenosine findings dissimilar from those commonly observed in pyruvate kinase deficiency hemolytic anemia. Heterozygous pyruvate kinase activity was clearly demonstrated in the mother and father as well as in certain relatives on both sides of the family. In addition, beta-thalassemia was found in the father and certain paternal family members. The possibility of joint inheritance of heterozygous pyruvate kinase deficiency and beta-thalassemia was considered, but it was not possible to demonstrate this with any certainty in the affected propositus.

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Extra Renal Production of Erythropoietin in Man

E. A. Mirand, G. P. Murphy, R. A. Steeves, H. W. Weber and F. P. Retief

Introduction

Erythropoietin (ESF) is a hormone active in the regulation of erythropoiesis and is activated or produced in response to hypoxia [3–9] Although the kidney has been postulated as the sole source of the production of this hormone [5] work by others has confirmed that there is experimental evidence for an extra-renal source [1 2 7 8, 9 13] This report confirms for the first time the presence of ESF release in an anephric human prior to renal homotransplantation.

Clinical History

The patient, a 10-year-old male, J v W., was admitted to the Karl Bremer Hospital in a terminal uremic state with glomerulonephritis in a solitary left kidney. Following initial peritoneal dialysis, abdominal exploration and left nephrectomy were undertaken. A thorough examination of the abdomen and pelvis confirmed the complete absence of the right kidney as was suggested by roentgenography and cystoscopic examinations. Hemodialysis was continued on a bi weekly schedule. A human cadaver renal homotransplant was performed after 66 days of the renoprival state. The kidney obtained from a patient also used as a human heart transplant donor was severely ischemic although some recovery was evident after the 13th postoperative day

Methods

Erythropoietin assay Plasmas from the patient were collected and frozen. All specimens were assayed at the same time in adult Ha/ICR Swiss mice rendered polycythemic [6, 10] Four to seven mice were used to determine the ESF activity for each plasma sample. Each test mouse received subcutaneous injections of 0.5 ml of plasma daily for 3 successive days. On the 4th day they were injected intravenously with 1 μg of ^{59}F in 0.5 ml saline. Twenty-four hours later they were bled from the dorsal aorta, and the radio-activity of the blood sample was measured in well-type scintillation counter The percentage incorporation of radio-active iron into the circulating red cells was then calculated [11]. Assays from mice with hematocrit levels of less than 60% at the end of the experiment were discarded.

Results

Erythropoietin levels Serial alterations in erythropoietin (ESF) are shown in tables I and II Plasma ESF levels obtained on the patient while in an anephric state for 66 days are higher than the range observed for the 24 hour ^{59}Fe uptake in control uninoculated polycythemic mice (2.6%) (table I) During the renoprival state the ESF levels in the patient ranged from 6.9 to 25.2%. It is interesting that the highest ESF level was obtained while the patient was anephric and that during this time the lowest hemoglobin level was recorded. Moreover the patient went over 25 days without blood transfusion during which time the highest level of ESF was reported (table I)

Table II shows ESF levels in the patient at various days post homotransplantation. Consistent high levels of ESF were observed during this period

No correlation was noted between the level of azotemia and the degree of ESF response in the renoprival state as is usually seen in patients with kidneys in a state of acute or chronic dysfunction (tables I and II)

Hematological observations The patient maintained a normochromic *microcytic anemia with minimal schistocytosis*, occasional burr cells, but normal platelet counts. Repeated transfusions were necessary to correct a rapidly falling hemoglobin, at least partially due to hemolysis of infection and glomerulonephritis. Before nephrectomy the reticulocyte counts varied between 1.5 and 3 1%, and after nephrectomy they ranged from 0.3 to 2.5% It was difficult to assess bone marrow potential in the clinically complicated anephric state, but bone marrow aspirate obtained shortly after renal transplantation was normocellular with a M E ratio of 3 1 and no other gross abnormalities were observed.

Table 1 Clinical course and plasma erythropoietin levels in patient while anephric

Days renoprival	Operative procedure	Hemodialysis	Blood transfusions	Hematocrit vol. %	Hemoglobin g %	Urea mg %	Plasma ESF assay (Average 24-h ^{59}Fe blood uptake in polycythemic mice†)
0	Left nephrectomy (right kidney absent)						S.E.
7		+					
19		+		26	12.9	108	8.6 ± 2.1
22		+	1 unit	18	5.7	109	
25		+		20	7.9	180	6.9 ± 0.9
28		+		20	6.3		14.8 ± 2.3
32		+		22	7.8	146	–
42		+		19	6.3	97	8.3 ± 0.1
46		+		19	5.6	154	25.2 ± 8.5
49		+	1 unit	30	11.1	150	–
53		+		19	5.7	53	12.2 ± 1.0
58		+		17	0.1	56	10.5 ± 1.0
60		+	1 unit	28	8.9	126	12.0 ± 0.2
63		+		24	7.4	67	11.8 ± 4.3
68	Kidney transplant		2 units			–	–

† 7 uninoculated polycythemic mice had a mean ^{59}Fe uptake of 2.6% ± 0.4

transplants, we noted release of ESF in response to acute ischemic and cellular destruction [12] Such may therefore, be the case in this patient.

Although it appears that an extra renal site, or sites, of ESF production does exist, this may be brought into play only after an animal or man has been nephrectomized. This might be analogous to the increased production of sex steroids by the adrenals of castrated animals and to the compensatory hypertrophy of the reticulo-endothelial system that follows splenectomy. Where the extra-renal site or sites for ESF production might be is unknown at this time, but perhaps the liver is one site. Despite our lack of knowledge of the site or sites, one can conclude that extra-renal production of ESF does occur in man as well as in other animals.

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Summary

Erythropoietin (ESF) is hormone active in the regulation of red blood cell formation. It has been commonly thought to originate only in the kidney and was believed to be activated or produced in the kidney in response to hypoxia. The human renal homotransplant patient described herein demonstrated that ESF activity occurs in the renoprival state. This observation in human confirms previous experimental evidence that extra-renal source of ESF does exist. Where this extra-renal source is located has not been determined either in animals or in man.

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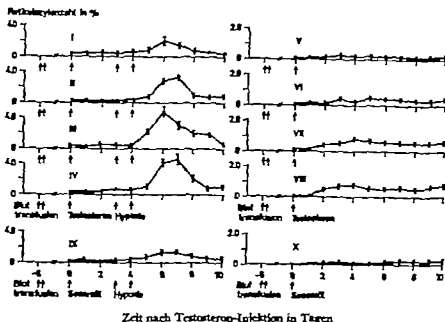


Abb. 1. Veränderung der Retikulozytenzahl im Schwanzvenenblut nach Testosteron-Injektion allein oder nach Testosteron-Injektion und hypoxischer Stimulation.

festgestellt. Bei allen Versuchstieren war die erythropoetische Reaktion höher als die Summe der Reaktionen, die jeder der Reize einzeln ergeben hatte. Die stärkste Reaktion wurde bei den Ratten beobachtet, die Testosteron 72 h vor der Hypoxie bekommen hatten, während die geringste Wirkung bei den Ratten auftrat, auf die beide Reize simultan einwirkten.

Diskussion

Es wurde festgestellt, dass die erythropoetische Reaktion auf kombinierte Reizung durch Testosteron und Hypoxie bei plethorischen Ratten, ausgedrückt durch den Anstieg der Retikulozytenzahl, die Summe der Einzelwirkungen beider Reize beträchtlich übertraf, ausgenommen die Ratten, die weniger als 0,5 mg Testosteron bekommen hatten. Dieses Ergebnis könnte als Beweis dafür interpretiert werden, dass Kombination von Hypoxie und Testosteron die Erythropoese verstärkt.

Es ist dabei von Interesse, dass diese synergistische Wirkung am deutlichsten dann erzielt wurde, wenn die Hypoxie 3 Tage nach der Testosteron Injektion einsetzte, während sie am wenigsten evident

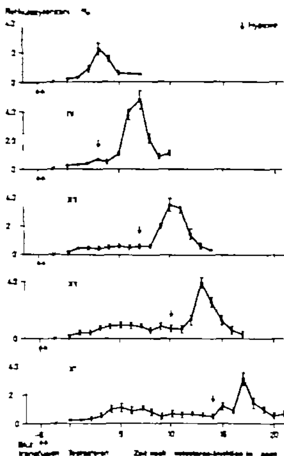


Abb. 2. Veränderung der Retikulozytenzahl bei Ratten der Gruppen IX, XI, XIV nach Testosteron-Injektion und hypoxischer Stimulation.

war bei den Ratten, die beide Reize gleichzeitig empfangen hatten. Der Anstieg der Retikulozytenzahl 48 h nach einer Einzeldosis von 5,0 mg langwirkendem Testosteron war klar erkennbar und erreichte sein Maximum nach 96 h. Von da an blieb die Retikulozytenzahl während der restlichen Versuchsdauer erhöht. Diese Ergebnisse zeigen, dass das Testosteron ziemlich schnell vom Injektionsort aus freigesetzt wird und seine stimulierende Wirkung auf die Erythropoese während der ganzen Beobachtungszeit behält. Es ist bekannt, dass Testosteron die Erythropoese bei verschiedenen Versuchstieren verstärkt, indem es die Bildung von Erythropoetin anregt [1–4].

Tabelle 1 Retikulozytenreaktion auf Hypoxie, Testosteron oder beide

Versuchsgruppe	Zahl der untersuchten Tier	Behandlung	Testosteron-Dosis, mg	Tage zwischen Testosteron-Inj. und hypox. Stimul.	Maximale Retikulozytenzahl %[1]	P
X	5	Sesamol	0		0,20 ± 0,08	–
V	5	Testosteron	0,5		0,32 ± 0 08	n.
VI	5	Testosteron	1 0		0 49 ± 0,06	< 0,01
VII	5	Testosteron	3,0		0,79 ± 0,15	< 0,001
VIII	5	Testosteron	5,0		0,79 ± 0,08	< 0,001
IX	5	Sesamol + Hypoxie	0	3	1,32 ± 0,36	
I	5	Testosteron + Hypoxie	0,5	3	1,98 ± 0,08	n.s.
II	5	Testosteron + Hypoxie	1,0	3	3,25 ± 0,34	< 0,001
III	5	Testosteron + Hypoxie	3,0	3	4,80 ± 0,91	< 0,001
IV	5	Testosteron + Hypoxie	5,0	3	4 70 ± 0 70	< 0,001
XI	5	Testosteron + Hypoxie	5,0	0	2,20 ± 0,49	–
XII	5	Testosteron + Hypoxie	5,0	7	3,[illegible]0 ± 0 48	< 0,01
XIII	5	Testosteron + Hypoxie	5,0	10	3,95 ± 0 42	< 0,001
XIV	5	Testosteron + Hypoxie	5,0	14	3,08 ± 0 45	< 0,05

Mittelwert Statistische Signifikanz zur Sesamol-Kontrollgruppe zur Sesamol- und Hypoxie-Kontrollgruppe oder zur Testosteron- und Hypoxie-Kontrollgruppe
n.s. nicht signifikant
± mittlere Standardabweichung des Mittelwertes

Die synergistische Wirkung von Testosteron und Hypoxie auf die Erythropoese ist an Mäusen schon von Gurney und Fried [3] nachgewiesen worden. Diese Autoren haben ausserdem berichtet, dass der synergistische Effekt zweier kleiner aufeinanderfolgender Dosen von Erythropoetin grösser ist als dies von den Einzeldosen zu erwarten wäre. Es könnte sich hier um einen synergistischen Effekt von Testosteron und Hypoxie auf die Blutbildung handeln. Die Autoren haben jedoch an anderer Stelle [4] darauf hingewiesen, dass männliche Mäuse aufgrund ihrer höheren Produktion an endogenem Testosteron nach hypoxischer Stimulierung mehr Erythropoetin bilden. Sie folgerten daraus, dass Testosteron Veränderung an den Nieren bewirken müsse, wodurch diese während der Dauer der Hypoxie mehr Erythropoetin als unter normalen Bedingungen ausscheiden. Es ist jedoch möglich, dass die Reaktion des Knochenmarkes auf die Hypoxie durch Testosteron verändert wird. Der Synergismus könnte durch eine komplexe Wechselwirkung von Testosteron und Hypoxie bedingt sein. Es bedarf weiterer Untersuchungen, um den dabei wirksamen Mechanismus aufzufinden.

Der Autor ist Herrn Doz. Dr. H. Klupp und Herrn Dr. R. Engelhorn sehr verpflichtet und dankt für die Erlaubnis zu dieser Arbeit, ebenso Herrn E. Weller für seine erfahrene technische Assistenz.

Zusammenfassung

Der synergistische Einfluss von Testosteron und Hypoxie auf die Erythropoese wurde an plethorischen Ratten bestätigt. Ein maximaler Anstieg der Retikulozyten im peripheren Blut als Folge der Hypoxie wurde bei Ratten beobachtet, denen 72 Stunden zuvor Testosteron injiziert worden war. Dabei war die Retikulozytenreaktion proportional der Menge von Testosteron.

Summary

A synergistic effect of testosterone and hypoxia on erythropoiesis was confirmed in plethoric rats. A maximal increase of reticulocytes in peripheral blood following hypoxia was observed in the rats injected with testosterone 72 h previously. In this case, the reticulocyte response was proportional to the amount of testosterone injected.

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Acta haemat. 39 373–380 (1968)

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Blastoid Transformation of Rabbit Peripheral Lymphocytes by Phytohemagglutinin Using a Microculture Technique

S. M. SABESIN

The potentiality of the mammalian small lymphocyte for growth and differentiation has been the object of considerable recent investigation [1] Studies with human peripheral lymphocytes have now repeatedly demonstrated that such cells may be transformed into cytologically immature blastoid cells capable of RNA and DNA synthesis and mitotic division when grown in suspension culture with phytohemagglutinin [2, 3 4] Recent reports [5 6 7 8] indicate that other agents, presumably acting as antigens, can also induce blastoid transformation in peripheral lymphocytes provided the cells have been obtained from specifically immunized donors.

The phenomenon of blastoid transformation thus provides a unique opportunity for investigating those factors which control the growth and differentiation of small lymphocytes and for studying the role of these cells in immune phenomena. Furthermore the recent observation [9 10] that peripheral lymphocytes from mammalian species, other than man, may also be transformed into blastoid cells by PHA affords a means of designing experimental animal models for the study of lymphocyte differentiation

Studies of blastogenesis in man and other species has usually involved the separation of lymphocytes from relatively large volumes of peripheral blood (50–100 ml) Whereas such quantities can easily be obtained from human subjects this requirement places a severe limitation on experiments involving small animals. Thus in our experience [9] sufficient blood for certain studies in the guinea pig could only be obtained by exsanguination thereby obviating the possibility of repeated cultures from the same donor

Attempts to utilize small quantities of blood for cytogenetic analysis were reported by Edwards and Young [11] Edwards [12] and Froland [13] In these studies adequate mitotic activity for cytogenetic analysis was obtained with as little as 0 1 ml of blood however the methods described involved the separation of leukocytes from whole blood by phytohemagglutinin (PHA) or dextran sedimentation of erythrocytes prior to the start of culture. The separatory procedures are time consuming but even more important do not allow for the complete use of the lymphoid population in the culture inocula since complete harvesting of the leukocytes cannot be obtained by erythrocyte sedimentation. Furthermore in certain mammalian species it is difficult, if not impossible, to obtain adequate erythrocyte sedimentation by ordinary methods.

In 1963 Arakaki and Sparkes [14] described a technique for culturing leukocytes from heparinized whole blood using minute samples of blood (0 05–0 1 ml) This method yielded satisfactory metaphase figures for complete chromosome analysis and there was no apparent modification of chromosome morphology due to the presence of whole blood elements throughout the period of culture.

In the present study lymphocyte blastogenesis, induced by PHA was studied in microcultures of whole blood using a technique similar to that described by Arakaki and Sparks [14] The efficacy of the method was evaluated by comparing blastoid transformation, and the kinetics of RNA and DNA synthesis in the microcultures with that obtained in lymphocyte cultures prepared from large volumes of blood by the usual techniques [15]

Materials and Methods

I these experiments lymphocytes were cultured by the micro and standard techniques from the same sample of blood. For this reason large volume (30 ml) of blood was drawn into heparinized collecting syringe by cardiac puncture of New Zealand white rabbits The microcultures were seeded by dispensing three drops of heparinized blood, directly from the collecting syringe into 16 × 125 mm screw-topped culture tubes previously prepared with 4 ml of complete culture media. The complete media was composed of Eagle's minimal essential medium, modified for suspension culture, supplemented with 20% fetal calf serum and 1% L-glutamine 200 mm. Phytohemagglutin (0 1 ml) Type M, Difco Labs, Detroit, Mich., was immediately added to each tube which were then tightly sealed and incubated vertically without agitation at 37° C. Alternatively the tops were loosely capped and the cultures incubated in humidified atmosphere of 5% carbon dioxide in air at 37° C. In this manner multiple replicates could be easily and very rapidly cultured from small volume of blood.

For the routine cultures 30 ml of heparinized blood was transferred to 45 ml centrifuge tube and incubated with one-half volume high molecular weight dextran

(250,000 m.w.) for about 20 min t 37°C to insure adequat erythrocyte sedimentation. The supernatant leukocyte-rich plasma was aspirated, centrifuged at 800 rpm for 10 min, washed twice with minimal essential medium nd diluted with complete medium to final concentration of 7.5×10^5 lymphocytes/ml. Four ml aliquots were inoculated into 16×125 mm screw cap culture tubes, 0.1 ml PHA, Type M added and the tubes incubated without agitation 37°C.

T study the sequential morphological changes induced by PHA, replicate micro and regular cultures were incubated for 2 4, 18, 24 48 and 72 h either with 0.1 ml PHA or in its absence The cultures were harvested by gentle centrifugation (800 rpm for 10 min) and the pellets resuspended in 1% sodium citrat for 2 min. The cells were then fixed in mixture of absolut methanol and glacial acetic acid (3 1) for 10 min, centrifuged again and then finally suspended in small olume of fixative. Air dried slides were stained with 1.0% acetic-orcein and examined by phase contrast microscopy The per cent of cytologically transformed cells and small lymphocytes, at each interval of culture was determined by averaging the differential counts of at least 1000 cells from each of two or more replicate cultures.

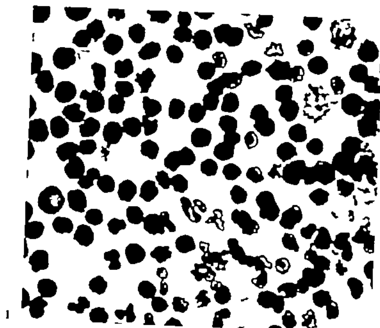
DNA and RNA synthesis were studied by autoradiography with ^3H-thymidine and ^3H-cytidine respectively Duplicate cultures at each time interval were gently agitated and then incubated for 2 h at 37°C with either ^3H-thymidine 6.7 /mm or ^3H-cytidine 2.68 curies/mm (New England Nuclear Corp., Boston, Mass.) added to the culture tubes at final concentration of 1.25 μc/ml.

The cells were then washed three times in phosphate buffered saline and fixed ten minutes with absolute methanol and glacial acetic acid (3 1) The slides were coated with Kodak NTB2 Nuclear Track Emulsion and stored for two weeks at 4 C. The autoradiographs were developed in D-72 Kodak developer and stained with Jenner Giemsa. The percent of labelled mononuclear cells in sample of 1000 cells on each slide was then determined.

Results and Discussion

In Table I the percent blastoid cells and the percent of lymphocytes labelled with H thymidine in the micro and routine cultures are compared at intervals throughout the 72 h of incubation At the beginning of culture the lymphoid cells in the inoculum of the microcultures were almost all typical small lymphocytes (fig 1) except for 0.4–1.5 larger cells which could be distinguished not only by size but also by their cytologically immature nuclei. Using phase contrast it was possible to distinguish extremely early blastoid transformation characterized by a change in appearance of the nuclear chromatin which assumed a delicate finely reticulated pattern in contrast to the dense clumped chromatin in the typical small lymphocyte

Evidence of blastoid transformation was apparent in the phytohemagglutinin stimulated micro and routine cultures within the first 24 h of incubation. As early as the 18th hour of culture there was a definite increase in the percentage of blastoid cells which now comprised 7 2–18.0% of the lymphocytes compared with



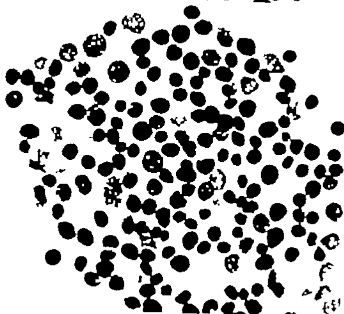


Fig. 1. Phase contrast micrograph of the cellular inoculum at the beginning of microculture. This field is composed predominantly of typical small lymphocytes which can be distinguished easily from the occasional larger lymphocyte by their uniform small size, scant cytoplasm and deeply staining nuclear chromatin.

Fig. 2. Phase contrast micrograph of lymphoid aggregate 18 h after the addition of phytohemagglutinin to the microcultures. Even by this time evidence of blastoid transformation is apparent in many lymphocytes characterized by nuclear and cytoplasmic enlargement, prominent nucleoli and pale-staining delicately reticulated nuclear chromatin.

0.9–2.0 in the cultures grown without PHA. In addition to the increase in the percent of transformed cells the lymphocytes were beginning to aggregate into small clusters and it was in these lymphoid foci that the earliest conclusive evidence of transformation could be detected (fig 2)

Autoradiography with H thymidine disclosed that blastoid transformation preceeded active DNA synthesis. There was only a slight increase in the number of lymphocytes labelled with ^3H thymidine at 24 h (1.5–2.0) over that obtained in the first 4 h of culture (0 6–1 0) In contrast RNA synthesis was active even in the small lymphocytes, throughout the entire duration of culture Within the first few hours of incubation up to 91 of the cells were labelled with ^3H-cytidine and when the cultures were terminated at 72 h between 92 and 98 were labelled.

The blastoid response proceeded vigorously after the first 24 h and by the end of the second day of incubation between 53 and 68 % of the cells had been transformed into blastoid cells compared with 0.8–4 0 % in the controls (table I)

Although there was a modest increase in the percentage of cells labelled with ^3H-thymidine at 48 h DNA synthesis could not be considered a prerequisite for transformation since only 4 to 8 of

Table I Blastoid transformation and DNA synthesis in micro and routine cultures of rabbit peripheral lymphocytes

Hours of incubation	Percent blastoid cells Microcultures	Percent blastoid cells Routine	Percent cells labelled ^3H-thymidine Microcultures	Percent cells labelled ^3H-thymidine Routine
0	0.4– 1.5	0.3– 2.4	0.2 0.8	0.2 0.5
2	0.4– 1.8	0.5– 2.1	0.8– 1.0	0.2 0.9
4	1.0– 2.1	2 1 2.3	0.6– 1.0	0.4– 0.5
18	7.2 18.0	17.0–20.0	0.2 2.0	0.5– 2.4
24	18.0–33.0	17.0–30.0	1.5– 2.0	1 5– 2.8
48	53 0–68.0	60.0–73 0	4.2 8.0	5 0– 8.0
72	71.0–80 0	60.0–88.0	15.0–30.0	15.0–40 0

Effect of phytohemagglutinin (PHA) on rabbit peripheral lymphocytes in micro and routine cultures. The data represent the range of values obtained for blastoid transformation and ^3H-thymidine incorporation at each interval of culture. Cultures for radioautography were incubated for 2 h with ^3H-thymidine specific activity 67 c/mM at final concentration of 125 μc/ml. Autoradiographs were exposed for 14 days with Kodak NTB2 Nuclear Track emulsion.

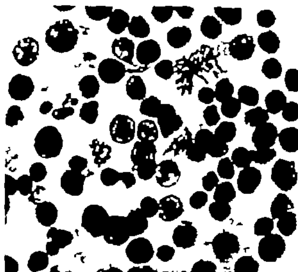


Fig 3. Phase contrast micrograph showing extensive blastoid transformation and mitosis in microculture 72 h after the addition of phytohemagglutinin.

the cells had incorporated the label at this time. When the cultures were terminated at 72 h between 71 and 80% of the cells were blastoid and now autoradiography disclosed a substantial increase in DNA synthesis with 15 to 30% labelled cells after a 2 hour incubation with ^{3}H thymidine. At 72 h control cultures contained 5 6–10 0% blastoid cells and of these 0.5–1 5 % were labelled with ^{3}H thymidine.

At 72 h the small lymphoid aggregates in which transformation was first noted, had enlarged and mitotic figures were evident (fig 3) The transformed cells showed considerable variation in size but were readily distinguished from the small lymphocytes by their prominent nucleoli and pale staining delicately reticulated nuclear chromatin.

The transformed cells in the microcultures were similar cytologically to the blastoid cells obtained in cultured peripheral lymphocytes grown at a concentration of 7 5 × 10^3 lymphocytes/ml. Furthermore the sequential cytological alterations induced by PHA and the kinetics of RNA and DNA synthesis in the transforming cells were approximately equivalent with both methods of culture (table I) In each instance significant blastoid transformation was evident by 24 h and this cytological alteration was not dependent upon prior DNA synthesis.

These studies indicate the feasibility of utilizing extremely small quantities of heparinized whole blood for studying lymphocyte blastogenic transformation. Our comparison of the microculture technique with replicate cultures prepared from the same sample of blood by the conventional technique suggests that under these cultural conditions the sequential transformation of small lymphocytes into blastoid cells capable of RNA and DNA synthesis and mitotic division is not affected by the presence in the inoculum of polymorphonuclear leukocytes, erythrocytes or other blood elements. These results confirm the previously published report of Arakaki and Sparks [14] who devised the method of microculture for use with heparinized whole blood obtained from mice.

The microculture technique affords an extremely rapid and simple means of culturing peripheral blood lymphocytes but should find its greatest potential usefulness in experimental situations in which repeated cultures from the same donor are required.

Acknowledgment

This work was supported in part by grants from the National Institutes of Health (AM-03014 and AM-4501) and the Medical Foundation, Boston, Mass.

Summary

A microculture technique is described for studying the blastoid transformation of peripheral blood lymphocytes utilizing 0.1 ml quantities of heparinized whole blood. This technique was compared with routine lymphocyte cultures using 3×10^6 lymphocytes/tube obtained by partial purification of dextran-sedimented whole blood. Blastoid transformation RNA and DNA synthesis were compared in the microcultures and in routine cultures seeded with lymphocytes obtained from the same blood specimen. The kinetics of RNA and DNA synthesis and the sequential cytological transformation of small lymphocytes by phytohemagglutinin was essentially the same in both methods of culture. Microcultures thus provide a simple and rapid technique for in vitro studies of lymphocyte growth and differentiation.

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Strahlenschäden und Strahlenhämatologie Vorträge aus einem Fortbildungslehrgang der Deutschen Akademie für ärztliche Fortbildung 1964 bearbeitet von A. MONCZEK. Schriftenreihe der ärztlichen Fortbildung, Band 31 herausgegeben von H. REDETZKY und H. THELE. VEB Verlag Volk und Gesundheit, Berlin 1965. 166 S., 70 Abb., 9 Tab. Preis DM 25.

Der Begriff der Strahlenhämatologie umschreibt einerseits Einwirkungen ionisierender Strahlen auf die Blutbildung und -zellen, andererseits die Anwendung radioaktiver Isotope in der Hämatologie für Diagnostik, Therapie und Forschung. Im vorliegenden Band finden sich Übersichtsreferate über diese beiden Aspekte welche in ihrer Gestaltung einem kurzgefassten Lehrgang für Mediziner und Biologen entsprechen. Einiges Interesse beanspruchen eigene Beobachtungen von K. H. KLARE und in einem weiteren Kapitel von A. MONCZEK über hämatologische Veränderungen bei therapeutisch bestrahlten Patienten. Die Diskussion der Behandlung der Polyzythämie mit Radiophosphor basiert teilweise ebenfalls auf eigenen Untersuchungen von E. W. DÖRFFEL, S. MÖRKERT und R. VOLLMAR. Die Autoren schätzen das Risiko dieser Behandlung als gering ein; sie beobachteten beispielsweise in einer Serie von 231 mit Radiophosphor behandelten Patienten nur 5 Fälle welche in eine Leukämie übergingen. Die Referate über das akute Strahlensyndrom, die Überwachung von strahlenexponierten Personen und die Anwendung radioaktiver Nuklide für diagnostische Zwecke enthalten nur wenig Angaben, welche nicht ausführlicher in den neueren Lehrbüchern der Nuklearmedizin auffindbar wären.

E. BECK, *Basel*

J. E. CLEAVER **Thymidine Metabolism and Cell Kinetics**; in Frontiers of Biology Vol. 6. North Holland Publ. Co., Amsterdam 1967 259 S. Preis Hfl. 45.

This book is an excellent example of the value of the introduction of new basic method in the field of biology Owing to the specificity of Thymidine for DNA, tremendous advances were made in the study of the kinetics of cell population by using radioactive derivatives of Thymidine After introduction on the preparation, stability and detection of labelled thymidine the author presents detailed data on the metabolism of thymidine the basic problems of cell kinetics with particular emphasis on the behaviour of chromosomal DNA. The book will serve as valuable textbook and very complete reference on this basic topic

P. FRICK, *Zürich*

Automation in Haematology Proceedings of symposium held at the XIth Congress of the International Society of Haematology Brit. J. Haemat., supplementum ad vol. 13 April 1967 Hrsg. S. M. LEWIS. 75 Seiten. Preis 21 sh.

Nachdem automatisierte Methoden in zunehmendem Masse auch in haematologischen Laboratorien Anwendungsbereiche finden und propagiert werden, erscheint eine kritische Standortbestimmung heute besonders wertvoll. Die vorliegende Zusammenstellung enthält Übersichtsreferate über die Planung Standardisierung und praktische Anwendung, aber auch erste Ergebnisse automatisierter Methoden, beispielsweise in der Blutgruppenserologie. Die Beiträge dieser kleinen Monographie können gesamthaft gewürdigt werden sie zeigen in klarer Form, dass eine teilweise Automatisierung in der klinisch orientierten und experimentellen Hämatologie in naher Zukunft unentbehrlich sein wird sie wird, bei sachgerechter und kritischer Anwendung, repetitive Arbeitsgänge rationalisieren, die qualitative Reproduzierbarkeit erhöhen und vor allem geschultes Personal zugunsten produktiverer Arbeit entlasten.

Die Automatisierung wird dann ihre volle Wirksamkeit erreichen, wenn sie nicht nur eine Rationalisierung der Arbeitsmethoden, sondern auch die Möglichkeit einer internationalen Standardisierung hämatologischer Untersuchungsmethoden mit sich bringt. Die in dem vorliegenden Heft enthaltenen Empfehlungen des «International Committee for Standardization in Haematology» sind richtungsweisend.

E. BECK, *Basel*

Varia

International Council of Nurses

Since August 1966, Geneva has been the headquarters of the International Council of Nurses, world-wide organization t which 63 national nurses associations are affiliated. The Council decided to transfer its headquarters from London to Geneva so as to maintain closer relations with the World Health Organization and similar international organizations with nursing interests.

The publication of the *International Nursing Review* official journal of the International Council of Nurses, is one of the Council' principle undertakings. Other publications include *ICN Calling* (the ICN news letter containing news of topical interest) and information leaflets in three languages, and other publications of professional interest.

In 1969 the International Council of Nurses – possibly the largest women professional association in existence – is holding an international congress in Montreal, which nearly 12,000 nurses from most parts of the world are expected to attend.

Deutsche Gesellschaft für Hämatologie

Auf der 13 Tagung der Deutschen Gesellschaft fur Hamatologie in Ulm/Donau wurde Prof. Dr K. LENNERT (Kiel) zum neuen Kongress-Präsidenten fur 1969 gewählt. Die nachst Tagung der Gesellschaft wird in der ersten Septemberwoche 1969 in Kiel stattfinden. Prof. Dr W STICH (Munchen) wurde für die Zeit von 1968–1970 zum Vorsitzenden der Gesellschaft wiedergewahlt. Dr K. G von BOROVICZÉNY (Freiburg/Breisgau) wurde zum neuen Sekretar der Gesellschaft gewählt

Auf der Tagung wurde ausserdem ein Ausschuss fur die Vorbereitung des XIII. Kongresses der Internationalen Gesellschaft fur Hamatologie gebildet, der 1970 in Munchen unter dem Präsidium von Prof. Dr L. HEILMEYER (Ulm) stattfinden wird.

II. International Symposium on Foeto-Maternal Incompatibility

Brussels, November 15 and 16, 1968

Round table on the Prophylaxis of Rhesus(D)-Immunisation by pregnancy with immunoglobulins.

Plenary session, subjects 1 Analysis f amniotic fluid (bilirubin, hormones, cells, proteins, etc.) 2 Differential diagnosis of neonatal icterus (blood group incompatibility breast feeding-icterus, erythrocyte anomalies, etc.)

Symposium Secretariat Dr C. VERMYLEN, Blood Transfusion Centre, O.-L. Vrouwestraat 42, *Leuven*, Belgium.

Index rerum ad Vol. 39

Bearbeitet von G. Boerre, Basel

(B) = Book review Buchbesprechungen Livres nouveaux

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